UNIVERSITÉ DE SHERBROOKE
Faculté de génie
Département de génie chimique et génie biotechnologique

BIOVALORISATION DU PETIT LAIT EN 2,3-BUTANEDIOL PAR FERMENTATION

Biovalorization of whey into 2,3-butanediol by fermentation

Thèse de doctorat
Spécialité : Génie Chimique

David FERNÁNDEZ GUTIERREZ

Jury: Pr. Michèle HEITZ – Université de Sherbrooke (Directrice)

Pr. Anne GIROIR FENDLER – Université Claude Bernard Lyon 1 (Co-directrice)

Pr. Nathalie FAUCHEUX – Université de Sherbrooke (Co-directrice)

Dr. Antonio ÁVALOS RAMÍREZ - Centre National en Électrochimie et en Technologie Environnementales, Shawinigan (Co-directeur)

Pr. Bernard MARCOS – Université de Sherbrooke (Rapporteur)

Pr. Luc MALHAUTIER – IMT Mines Alès (Évaluateur externe)

Pr. José Luis VALVERDE PALOMINO – Universidad de Castilla La Mancha, Ciudad Real (Évaluateur externe)

Dr. Claude DESCORME – Université Claude Bernard Lyon 1 (Évaluateur externe)

Sherbrooke (Québec) Canada July 2018

Essentially to Isa, who left everything back to support me

RÉSUMÉ

Le lactosérum est un résidu liquide obtenu lors de la fermentation du fromage. Il est composé de lactose, de protéines, etc. Étant donné les valeurs élevées de ses demandes biologique (DBO) et chimique (DCO) en oxygène, il est nécessaire de traiter le lactosérum avant de le rejeter. La valorisation du lactosérum par voie biotechnologique devrait permettre a) de réduire la DBO et la DCO et b) de générer des produits tels que le 2,3-butanediol (2,3-BD). Des bactéries comme *Enterobacter cloacae* et *Klebsiella pneumoniae* peuvent hydrolyser et transformer des saccharides (ex. lactose) en 2,3-BD. Ces bactéries quoique pathogènes sont considérées comme étant les plus performantes pour générer du 2,3-BD. Par conséquent, l'utilisation d'autres souches bactériennes non pathogènes permettant l'hydrolyse et la fermentation des saccharides est recherchée. Dans ce contexte, une souche bactérienne non pathogène d'*Escherichia coli* capable d'hydrolyser et de fermenter une large variété de saccharides pourrait être une bonne option. Cependant, la souche *E. coli* ne produit pas de 2,3-BD. Une modification génétique d'*E. coli* a été effectuée afin de transférer la voie métabolique d'un producteur naturel de 2,3 BD à savoir l'*E. cloacae.* Par conséquent, l'objectif principal de cette étude est de valoriser le lactose (un disaccharide) contenu dans le lactosérum par fermentation en présence d'une souche génétiquement modifiée d'*E. coli*, afin d'obtenir du 2,3-BD. Un perméat de lactosérum a également été utilisé comme substrat. Dans un premier temps, une étude portant sur la valorisation potentielle du lactosérum en 2,3-BD a été réalisée. Les différentes bactéries et substrats utilisés pour produire du 2,3-BD sous différentes conditions opératoires sont détaillés. Dans un deuxième temps, la souche d'*E. coli* (*E. coli* JFR12) a été utilisée en variant la nature et la concentration du substrat dans le milieu de culture M9 (préalablement choisi comme le plus approprié parmi 4 milieux de culture). Le rendement en 2,3-BD le plus élevé (environ 0.36 g 2,3-BD/g saccharide) a été obtenu en présence de 25 g/L de glucose ou de lactose. Quelle que soit la concentration de galactose, les rendements en 2,3-BD étaient faibles. En présence du mélange glucose-galactose, les rendements en 2,3-BD obtenus étaient similaires à ceux obtenus avec le galactose. Ensuite, des mélanges de lactosérum ou de perméat de lactosérum ont été réalisés. Les fermentations de ces mélanges en absence de M9 améliorent le rendement en 2,3-BD jusqu'à 0.43 et 0.42 g 2,3-BD/g lactose en utilisant du lactosérum ou du perméat de lactosérum respectivement pendant 72 h. Finalement, l'effet des trois paramètres suivants (quantité d'inoculum, pH initial et vitesse d'agitation) ont été évalués. Les rendements en 2,3-BD les plus élevés ont été respectivement de 0.47 et de 0.44 g 2,3-BD/g lactose pendant 72 h en utilisant du lactosérum ou du perméat de lactosérum respectivement. Enfin, l'augmentation du volume réactionnel en utilisant un bioréacteur de 2 L pour la fermentation du lactosérum et du perméat de lactosérum a été testée en condition anaérobie. Les rendements en 2,3-BD ont été plus faibles par comparaison avec ceux obtenus à partir des expériences réalisées dans des fioles de 0.5 L. L'ajout d'air (2 L/min) dans le bioréacteur a été testé afin d'étudier son impact. Le rendement en 2,3-BD a été de 0.40 g 2,3-BD/g de lactose en 24 h. Le temps de fermentation a également été réduit de 72 h à 24 h. La souche *E. coli* JFR12 est appropriée pour valoriser le lactosérum et le perméat de lactosérum en 2,3-BD.

Mots-clefs: *Escherichia coli*, 2,3-butanediol, glucose, lactose, lactosérum, perméat de lactosérum.

ABSTRACT

Whey is a dairy effluent generated during the cheese manufacturing. It contains lactose (the main part of the dry matter of whey), proteins, etc. Due to its high biological (BOD) and chemical (COD) oxygen demands, it is necessary to treat the whey before releasing it in the environment. The valorization of whey via biotechnology will have to allow for a) a decrease of the BOD and COD values, and b) of a generation of products like 2,3-butanediol (2,3-BD). Bacteria like *Enterobacter cloacae* and *Klebsiella pneumoniae* are able to hydrolyze and transform saccharides like lactose into 2,3-BD. Although these bacteria are pathogenic, they are considered as the best 2,3-BD producers. Therefore, the use of other non-pathogenic bacterial strains is being developed for the hydrolysis and the fermentation of saccharides. In this way, a non-pathogenic strain of *Escherichia coli* able to hydrolyze and ferment a wide range of saccharides would be a good option. However, *E. coli* cannot produce 2,3-BD. A genetic modification in the *E. coli* strain has been performed in order to transfer the metabolic pathway from a natural 2,3-BD producer: *E. cloacae*. In this way, the main objective of this research is to valorize the lactose (a disaccharide) contained in the whey via fermentation in the presence of a genetically modified strain of *E. coli* K12 MG1655 in order to obtain 2,3-BD. Permeate whey, was also used as a substrate. Firstly, a literature review about the potential valorization of whey into 2,3-BD was performed to show the different kinds of bacteria and substrates used to produce 2,3-BD under different operating conditions. Secondly, the *E. coli* strain (*E. coli* JFR12) was used varying the substrate nature and concentration in M9 culture medium (previously chosen as the most appropriate among 4 culture media). The highest 2,3-BD yield (around 0.36 g 2,3-BD/g saccharide) was obtained in the presence of 25 g/L of glucose and lactose. Whatever galactose concentration used, low 2,3-BD yields were obtained. In the presence of a mixture of glucose-galactose (1:1, w/w), the 2,3-BD yields were similar to those obtained using galactose as a sole carbon source. Afterwards, three mixtures of whey or permeate whey in the presence or absence of M9 culture medium were used. Fermentations of these mixtures in absence of M9 improved the 2,3-BD yield up to 0.43 and 0.42 g 2,3-BD/g lactose for 72 h using whey and permeate whey, respectively. Thirdly, the effect of three operating conditions (inoculum size, initial pH and agitation rate) was evaluated. The highest 2,3-BD yields were 0.47 and 0.44 g 2,3-BD/g lactose for 72 h using whey and permeate whey, respectively. Finally, an increase of the reaction volume using a 2 L bioreactor to ferment whey and permeate whey was tested under anaerobic conditions. The 2,3-BD yields under anaerobic conditions were lower compared to those obtained in flask of 0.5 L. On the other hand, the addition of air (2 L/min) in a 2 L bioreactor was tested in order to study its effect on 2,3-BD production fermenting whey. The 2,3-BD yield was 0.40 g 2,3-BD/g lactose at 24 h. Therefore, the fermentation was improved using 2 L/min of air in the bioreactor since a similar 2,3-BD yield was obtained in comparison to the one in 0.5 L flasks, reducing the fermentation time from 72 h to 24 h. The *E. coli* JFR12 strain is a suitable strain to valorize whey and permeate whey into 2,3-BD.

Keywords: Escherichia coli, 2,3-butanediol, glucose, lactose, whey, permeate whey.

ACKNOWLEDGMENT

Firstly, I would like to mention my gratitude to Professor Michèle Heitz since without her nothing of this could be possible. All her help and patience have been a key factor during this period and I have been very comfortable working, studying and learning at Université de Sherbrooke. In addition, I would like to emphasize the good relationship, the pleasant treatment with her.

Beside her was Professor Nathalie Faucheux, my co-director. I am very grateful to her for everything. Above all, I would like to thank her availability for talking with me in her office without any appointment. It has been a huge pleasure to be under her supervision. Her point of view about this topic has been very useful and I really appreciate all the time with her.

Another outstanding place in my mind and heart is for my second co-director, Dr. Antonio Ávalos Ramírez. His priceless help, his valuable suggestions, comments and his incredible professionalism have been a great support for me.

For all of them, I would like to thank all of that I am since I know that I have not been an easy student. Seriously, one thousands of thanks.

I would like also to thank my co-director of the joint PhD agreement Professor Anne Giroir-Fendler for her warm welcome at Université Claude Bernard Lyon 1. It has been formidable to be able to meet her and her good humor.

Beside my directors, I want to thank Marc Veillette his appreciated help and time. It has been very easy to discuss and work with him in order to present the results. Another special mention is for my lovely friends Mauricio González, Jennifer Gil, Sergio Croquer, Beatriz Caraballo, Edwin Rodriguez, Julie Martel, Shant Shahbikian, Marie Lupien-Durocher, Iulian Zoltan, Judit Hunyadkurti, Jean Baptiste “butterfly” Beigbeder, Pedro Durao, Milad Ferdowsi, Bahman Khabiri, Jamie Sharp, Rajesh Guruswamy, Amirjalal Jalali, Tim Spitters, Luc Treutens, Graciela Chavez and many other friends.

In addition, I also wish to thank nice and helpful technicians and unforgettable friends: Isabelle Arsenault and Valérie Larouche. Moreover, I would like to thank secretaries of the chemical engineering department at Université de Sherbrooke: France Auclair, Louise Carbonneau, Louise Chapdelaine and Sylvie Lebrun for their nice behaviors and helps.

Last but not the least, I would like to thank my family: my partner, Isa Aceña; my parents, Pedro Pablo and Rosa María; my brother and sister-in-law, Pedro and Zinthia; my uncle and cousin, Federico; my aunt, Catalina; my grandparents, Justo and Guadalupe; my parents-in-law, Julián and Alicia; and my uncle and aunt-in-law, Pepe and Susana, for their unconditional support and motivation during my whole life especially these 4 years. Finally, I would like to mention special friends from Spain: Beatriz Delgado, Erika Ligero, Gloria Chamorro, Jose Manuel Ruiz, Fernando López-Rey and Pilar López because on an adventure like this one, the most important thing is not to be far, it is to have people who make you feel close whatever the distance is.

Table of content

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1. Introduction

In a world more and more industrialized to cover all the human needs, the environmental issues are getting a special attention since the industrialization is causing negative environmental impacts, which is trying to be contained by, for instance, international environmental agreements. It is possible to underline among the human requirements, the basic consumer goods like milk and bread, and their production have to be increased because of the rise of the worldwide population. The industrialization causes the increase of greenhouse gases (GHG) like carbon dioxide (CO_2) (Panesar et al., 2007). However, the upgrades of the productive processes have reduced the GHG emissions. For example, in the dairy industry, 1.63 kg CO_2 equivalent per L of milk cow produced were generated in 1961; whereas this amount was reduced to 0.92 kg CO_2 equivalent per L of milk cow produced in 2014 (FAO-FAOSTAT, 2017a).

The dairy industry example is important since it is one of the most important industries in the agro-food sector. The main dairy product is the milk, which worldwide production was 791.8 million tons in 2014 (FAO-FAOSTAT, 2017b). However, all of amount of milk produced is not only allocated for human consumption but also for manufacturing other products derived from milk like cheese. Cheese production, an important dairy processed product in terms of volume, needs around 10 L of milk to make 1 kg of cheese (BCB, 2017). During the cheese manufacturing, a liquid effluent is generated: the whey (W). Whey is the liquid obtained after coagulating the milk and is generated at a rate of 9 L per kg of cheese manufactured. If it is directly released into any body of water, these important quantities of W can affect the aquatic life of rivers, lakes, etc. (De Jesús et al. 2015, Guimarães et al. 2010). Lactose, a disaccharide formed of glucose and galactose (monosaccharides), is the main dried solid compound of W with a concentration ranged between 46 and 54 g/L of W depending on the kind of W: sweet or acid (Hernández-Rojas and Vélez-Ruíz 2014, Panesar et al. 2007, Pescuma et al. 2015, Tsakali et al. 2010). One of the treatments that can be applied on W is its deproteinization by ultrafiltration (membranes), obtaining another liquid effluent: permeate whey (PW) (de Wit 2001). Permeate whey can present a lactose concentration higher than 80 g/L (Smith et al. 2016).

Lactose can be used in order to produce hydrogen (H_2), biogas, acetoin (A) and 2,3-butanediol (2,3-BD) by catalytic reactions performed by enzymes, commonly named "fermentation" (Pescuma et al., 2015; Roncal et al., 2010). The fermentation is performed in the presence of bacteria and under controlled operating conditions. Several parameters such as temperature, pH, agitation, kind of bacteria and concentration of substrate, can affect the yield of the fermentative products.

To the author's best knowledge, no study discussed the fermentation of the lactose content in W and PW by a modified strain of *Escherichia coli* in order to obtain 2,3-butanediol. In addition, no study discussed the transformation of galactose as a sole carbon source by fermentation in the presence of an *E. coli* strain.

The objective of this research was the transformation of lactose contained in the W and PW into 2,3-BD by a genetically modified strain of *Escherichia coli* (ECGM), which hosts the metabolic pathway of 2,3-BD from *Enterobacter cloacae*. During this research, the ECGM was improved in terms of the 2,3-BD production. In this way, two ECGM strains were used: *E. coli* JFR1 and *E. coli* JFR12. To the best of our knowledge, this is the first time that a genetically modified strain (ECGM) has been used in order to catalyze the W and PW fermentation.

This study was carried out under seven specific objectives:
1) The inoculum size of *E. coli* JFR1 before performing fermentations in order to obtain the highest bacterial population;
2) The effect of three glucose (used as a control) concentrations supplementing four culture media in the presence of *E. coli* JFR1 on the formation of A and 2,3-BD (ABD);
3) The influence of the type and concentration of an additional nitrogen source (ANS) in a defined culture medium (M9) on the ABD production using *E. coli* JFR1;
4) The 2,3-BD yield fermenting three concentrations of glucose, galactose and lactose in M9 culture medium with *E. coli* JFR12;
5) The effect of the lactose dilution contained in W and PW on the 2,3-BD yield in the presence of M9 (W:M9 or PW:M9), varying the ratio substrate/M9 (50:50, 75:25 and 100:0);
6) The influence of the inoculum size, the initial pH and the agitation rate fermenting W and PW in absence of M9 on the 2,3-BD yield;
7) The effect of air addition (2 L/min) on the 2,3-BD yield in a 2 L bioreactor in the presence of *E. coli* JFR12 and W.

This thesis contains 5 chapters including one review article and two research articles. In addition, one short paper is included as Annex 1.

Chapter 1 presents a general statement about the theme of the thesis. In this chapter, the whey and its permeate are introduced besides the objectives of the project.

Chapter 2 presents the state of art about the potential use of W in order to produce 2,3-BD. In this chapter, the W generation and its effects on the environment are introduced. In addition, the diverse kinds of treatments that can be performed on W in order to valorize it are discussed. Among these treatments, the biological process and particularly the transformation of several saccharides into 2,3-BD by different bacteria and how the operating conditions such as type and concentration of substrate, temperature, pH, agitation, etc. affect the 2,3-BD yield is also discussed.

Chapter 3 presents the estimation of the inoculum size by two experimental techniques: optical density (OD) and colony-forming unit (CFU). The wild and a genetically modified strain of *Escherichia coli* were characterized. Once the inoculum size was estimated, four culture media (LB, M9, M63 and MOPS) were tested at three glucose concentrations (4, 12.5 and 25 g/L) in the presence of the genetically modified *E. coli* strain (*E. coli* JFR1) to produce ABD. The culture medium M9 was selected according to the yield of ABD and to the cost of the culture medium. The chosen culture medium, M9, was supplemented with two ANS (sodium nitrate: $NaNO_3$, and urea: $(NH_2)_2CO$) and tested in the presence of 12.5 and 25 g/L of glucose. After

performing the analysis, it was confirmed that urea presented a positive effect on the ABD yield and the nitrogen content could be optimized.

Chapter 4 presents the 2,3-BD yields fermenting W and PW in the presence of an optimized *E. coli* strain: *E. coli* JFR12. Firstly, the fermentation of glucose, galactose and lactose at three concentrations (12.5, 25 and 50 g/L) in M9 culture medium supplemented with 15 g/L of urea (7.0 g N/L) was studied. The 2,3-BD yields as a function of the saccharide nature are discussed. Moreover, a mixture of glucose and galactose at two concentrations (12.5 and 25 g/L of each monosaccharide) was used as a substrate in the fermentation. Further, W1 and PW1 were mixed with M9 as follows: 50:50, 75:25 and 100:0 (v/v) and evaluating the fermentation process. After that, the effect of inoculum size (5.0, 7.5 and 10.0%, v/v), initial pH (6.5, 7.0 and 7.4) and agitation ratio (50, 100 and 200 rpm) on 2,3-BD production were evaluated using W1 and PW1. Finally, a new batch of whey (W2) and permeate whey (PW2) with a lactose concentration of 51 and 47 g/L, respectively, were tested in 0.5 L flasks and in a 2 L bioreactor (without aeration). The effect of the air addition (2 L/min) in a 2 L bioreactor (microaerobic conditions) on the 2,3-BD yield was tested using W2.

Chapter 5 is the conclusion of the thesis including the general findings of the project. Future perspectives are presented taking into account the found problems and the first obtained results.

1.1 Introduction in French

Afin de répondre aux besoins des populations, l'industrialisation s'accroît ce qui provoque une augmentation de la pollution. Les besoins humains et notamment la production de produits de consommation courante ne cesse d'augmenter en lien avec la croissance de la population mondiale. Par conséquent, des problématiques environnementales apparaissent suite à une industrialisation intensive qui se traduit par une augmentation des émissions de gaz à effet de serre (GES) comme le dioxyde de carbone (CO_2) (Panesar et al. 2007). Cependant, l'optimisation des procédés a permis de réduire les émissions de GES notamment dans le domaine de la production laitière. Par exemple, dans l'industrie laitière, un litre de lait produit, émettait 1.63 kg de CO_2 en 1961 alors que cette quantité était de 0.92 kg de CO_2 équivalent par litre de lait produit en 2014 (FAO-FAOSTAT, 2017a).

L'exemple de l'industrie laitière est important car il représente un secteur fort de l'industrie agroalimentaire. Le lait est le principal produit de cette industrie avec une production mondiale atteignant 791.8 millions de tonnes en 2014 (FAO-FAOSTAT, 2017b). Cependant, cette production annuelle se répartit entre le lait de consommation courante et les produits dérivés comme les fromages. La production de fromage, un important produit dérivé en termes de volume, nécessite 10 litres de lait par kilogramme de fromage produit (BCB, 2017). Au cours de la fabrication du fromage, un effluent liquide est généré: le lactosérum (W). Le W est le liquide résiduel issu du processus de coagulation du lait; 9 litres de W sont générés par kg de fromage manufacturé. En cas de rejets directs dans les eaux naturelles, ces quantités importantes de lactosérum peuvent nuire à la vie aquatique des fleuves, des lacs, etc. (De Jesús et al. 2015, Guimaraes et al. 2010). Le lactose, un disaccharide formé de glucose et galactose

(monosaccharides), est le principal composant de la fraction sèche du W avec une concentration variant entre 46 et 54 g/L de W, selon le type de W: sucré ou acide (Hernández-Rojas and Vélez-Ruíz 2014, Panesar et al. 2007, Pescuma et al. 2015, Tsakali et al. 2010). L'un des traitements pouvant être appliqué sur le W est sa déprotéinisation par ultrafiltration (membranes) afin d'obtenir du perméat (PW). Le PW peut avoir une concentration de lactose supérieure à 80 g/L (Smith et al. 2016).

Le lactose peut être utilisé pour la production d'hydrogène (H_2), de biogaz, d'acétoïne (A) et de 2,3-butanediol (2,3-BD) lors de réactions de catalyse enzymatique, appelée couramment "fermentation" (Pescuma et al., 2015; Roncal et al., 2010). La fermentation est effectuée en présence de bactéries et sous conditions contrôlées. Plusieurs facteurs comme la température, le pH, l'agitation, le type de bactéries, la nature et la concentration du substrat, peuvent modifier le rendement des produits de la fermentation.

À la connaissance de l'auteur, aucune étude n'a discuté de la fermentation du lactose présent dans W et PW par une souche modifiée d'*Escherichia coli* afin d'obtenir du 2,3-butanediol. En outre, aucune étude n'a examiné la transformation du galactose comme l'unique source de carbone par fermentation en présence d'une souche d'*E. coli*.

L'objectif de ce travail de recherche a porté sur la transformation du lactose contenu dans le W et le PW en 2,3-BD par une souche d'*Escherichia coli* génétiquement modifiée (ECGM). Tout d'abord, l'ECGM a été évaluée puis améliorée afin d'optimiser la production du 2,3-BD. Pour cela, deux souches d'ECGM ont été sélectionnées: *E. coli* JFR1 et *E. coli* JFR12. À notre connaissance, c'est la première fois qu'une souche génétiquement modifiée (ECGM) est utilisée pour la fermentation du W ou du PW.
Lors de cette étude, les sept objectifs spécifiques suivants ont été évalués:
1) la taille de l'inoculum d'*E. coli* JFR1 nécessaire pour réaliser des fermentations;
2) l'effet de trois concentrations de glucose et de quatre milieux de culture en présence d'*E. coli* JFR1 sur la formation d'A et de 2,3-BD (ABD);
3) l'influence du type et de la concentration d'une source additionnelle d'azote (SAA) sur le rendement en ABD dans un milieu de culture défini (M9) en présence d'*E. coli* JFR1;
4) le rendement en 2,3-BD lors de la fermentation de trois concentrations de glucose, de galactose et de lactose dans le milieu de culture M9 en présence d'*E. coli* JFR12;
5) l'influence de la dilution du lactose contenu dans W et PW sur le rendement en 2,3-BD en mélangeant W ou PW avec M9 (W:M9 ou PW:M9) et en variant le ratio substrat/M9 (50:50, 75:25 et 100:0 (v/v));
6) l'influence du volume d'inoculum, du pH initial et de la vitesse d'agitation lors de la fermentation du W et du PW en absence de M9 sur le rendement en 2,3-BD;
7) l'effet de l'oxygène (2 L/min d'air) sur le rendement en 2,3-BD lors d'une fermentation dans un bioréacteur de 2 L en présence d'*E. coli* JFR12 et de W.

Ce manuscrit de thèse contient 5 chapitres incluant un article de revue et deux articles de recherche. Un article complémentaire est annexé à ce document.

Le chapitre 1 est consacré à l'introduction du travail de recherche avec notamment des données relatives au lactosérum et au perméat. Les objectifs de l'étude sont ensuite précisés.

Le chapitre 2 présente l'état de l'art dans le domaine concerné et porte principalement sur la production du 2,3-BD à partir de W. Dans cette partie, la production de W et de ses effets sur l'environnement sont présentés. En outre, les différentes valorisations du W sont développées. Parmi ces traitements, le procédé biologique et en particulier la transformation de plusieurs types de saccharides en 2,3-BD par diverses souches bactériennes et les conditions d'opération (type et concentration de substrat, température, pH, vitesse d'agitation, etc.) sont discutés en détail.

Le chapitre 3 est consacré à l'estimation de la taille de l'inoculum. Deux techniques expérimentales ont été comparées : la densité optique (DO) et la méthode des unités formatrices de colonies (UFC). Les souches sauvage et génétiquement modifiée d'*E. coli* ont été caractérisées. Une fois la taille initiale de l'inoculum estimée, 4 milieux de culture (LB, M9, M63 et MOPS) ont été utilisés avec trois concentrations de glucose (4, 12.5 et 25 g/L) en présence de la souche d'*E. coli* génétiquement modifiée (*E. coli* JFR1). Le milieu de culture M9 fut sélectionné puisque le rendement en ABD était le plus élevé sur ce milieu et le coût de M9 était le plus faible. Le milieu de culture M9, a été complémenté avec deux sources additionnelles d'azote (SAA) (nitrate de sodium: $NaNO_3$, et urée : $(NH_2)_2CO$) et a été évalué en présence de 12.5 et 25 g/L de glucose. Il a été constaté que l'urée avait un effet positif sur le rendement en ABD; la teneur en azote a ensuite été optimisée.

Le chapitre 4 présente les rendements en 2,3-BD lors de la fermentation de W et PW en présence d'une souche d'*E. coli* optimisée: *E. coli* JFR12. Dans un premier temps, la fermentation du glucose, du galactose et du lactose (concentrations de 12.5, 25 et 50 g/L) dans le milieu de culture M9 enrichi de 15 g/L d'urée (7.0 g azote/L) a été étudiée. Les rendements en 2,3-BD en fonction de la nature du saccharide sont discutés. En complément, un mélange de glucose et de galactose sous deux concentrations (12.5 et 25 g/L pour chaque monosaccharide) a été réalisé puis utilisé comme substrat dans la réaction de fermentation. Enfin, un mélange de W1 (lot un (1) de lactosérum avec une concentration en lactose de 31 g/L) et de PW1 (lot un (1) de perméat avec une concentration en lactose de 34 g/L) a été ajouté au milieu de culture M9 dans les proportions suivantes: 50:50, 75:25 et 100:0 (v/v). L'effet du volume d'inoculum (5, 7.5 et 10.0%, v/v), du pH initial (6.5, 7.0 et 7.4) et de la vitesse d'agitation (50, 100 et 200 rpm) sur le rendement en 2,3-BD a été étudié lors de la fermentation des mélanges. Finalement, un nouveau lot de lactosérum (W2) et de perméat de lactosérum (PW2) avec une concentration de lactose de 51 et 47 g/L, respectivement, ont été fermentés en erlenmeyers de 0.5 L et en bioréacteur de 2 L (conditions anaérobies). L'effect d'air (2 L/min) en bioréacteur de 2 L (conditions micro-aérobies) sur le rendement en 2,3-BD a été testé avec W2.

Le chapitre 5 est la conclusion du travail de thèse. Des perspectives prenant en compte les difficultés rencontrées et les premiers résultats obtenus sont présentées.

CHAPTER 2. Literature review

Avant-propos:

L'article "Biovalorization of saccharides derived from industrial wastes such as whey – A review*"* a été publié dans le Journal "*Reviews in Environmental Science and Bio/Technology*" 16(1) (2017) 147-174.

TITRE: Biovalorisation des saccharides dérivés des déchets industriels tels que le petit lait – Un revue de la littérature.

Title: Biovalorization of saccharides derived from industrial wastes such as whey – A review.

David Fernández-Gutiérrez [a,b], Marc Veillette [a], Anne Giroir-Fendler [b], Antonio Avalos Ramirez [c], Nathalie Faucheux [a] and Michèle Heitz [a, d]

[a] Université de Sherbrooke, Department of Chemical Engineering and Biotechnological Engineering. Faculty of engineering, 2500 boul. de l'Université, Sherbrooke, (Québec), Canada, J1K 2R1

[b] Université Lyon 1, CNRS, UMR 5256, Institut de Recherche sur la Catalyse et l'Environnement de Lyon (IRCELYON), 2 avenue Albert Einstein, 69626 Villeurbanne, France

[c] Centre National en Électrochimie et en Technologies Environnementales, Shawinigan, (Québec), Canada, G9N 6V8

[d] Corresponding author: E-mail address: Michele.Heitz@USherbrooke.ca

Contribution to the document: This paper presents a literature review on the generation of whey issued from the cheese manufacturing. Whey can cause diverse environmental issues due to its composition, which depends on the kind of whey. Performing fermentation of whey in order to produce 2,3-butanediol is a solution to avoid the environmental problems caused this kind of biomass. A fermentation process depends on different factors like the kind and concentration of the carbon source, temperature, pH, agitation, etc. Among the operational parameters, one of the most important is the kind and strain of bacteria. In the review, these parameters are presented. Therefore, this review paper is helpful and relevant to the thesis topic.

Biovalorization of saccharides derived from industrial wastes such as whey – A review

2.1 Résumé

Le lactosérum est un résidu liquide résultant de la transformation du lait en fromage. Le lactosérum pose un problème environnemental majeur pour l'industrie laitière dû à sa charge organique élevée reliée à sa forte teneur en lactose.

La valorisation du lactosérum peut se faire par des procédés biologiques basés sur la fermentation du lactose en différents produits comme l'acide lactique (utilisés comme additif alimentaire), le 2,3-butanediol (matière première pour obtenir des produits comme la méthyl-éthyl-cétone utilisée dans les industries pharmaceutique et chimique), le biogaz (pour obtenir de l'énergie). La production de 2,3-butanediol à partir de saccharides tel que le glucose a été étudiée depuis plusieurs années en utilisant plusieurs sortes de micro-organismes comme *Enterobacter aerogenes*, *Panibacillus polymyxa*, *Klebsiella* sp., *Serratia marcescens* et *Escherillia coli* dont certains ont été génétiquement modifiés pour améliorer la production de 2,3-butanediol. Le potentiel de fermentation du lactosérum en 2,3-butanediol dépend de plusieurs paramètres opératoires comme la nature des micro-organismes, la composition du milieu de culture, la température, le pH et la teneur en oxygène.

Cet article de revue présente, tout d'abord, un bilan de la production du lait et du fromage au Canada et dans le monde. Puis, il décrit les différentes variétés de lactosérum et leurs techniques de traitement. Enfin, cet article présente la production par catalyse enzymatique du 2,3-butanediol à partir de divers saccharides en présence de microorganismes sous différentes conditions.

Mots-clefs: Industrie laitière, petit lait, microorganismes, 2,3-butanediol, conditions d'opération, fermentation.

2.2 Abstract

Whey is a liquid waste issued from the transformation of milk into cheese. Whey is a major environmental problem for the dairy industry due to its high organic load, linked to its high content of lactose. It can be valorized by biological processes based on lactose fermentation into different products such as (1) lactic acid (as food additive), (2) 2,3-butanediol (as feedstock to get products such as methyl-ethyl-ketone or 2-butene for the pharmaceutical and chemical industries), (3) biogas (to obtain energy). The production of 2,3-butanediol from saccharides, such as glucose, has been actively studied over previous decades using several types of microorganisms such as *Enterobacter aerogenes*, *Paenibacillus polymyxa*, *Klebsiella* sp., *Serratia marcescens* and *Escherichia coli*. Some of these have even been genetically modified to improve the 2,3-butanediol production. The potential whey fermentation process into 2,3-butanediol depends on several operating conditions such as microorganisms, composition of the culture medium, temperature, pH and aeration. This review first presents a summary of the situation of milk and cheese production in Canada and around the world. It also describes the different kinds of whey and their treatment techniques. Finally, this paper

describes the production of 2,3-butanediol from saccharides by various microorganisms under different operating conditions.

Keywords: Dairy industry, whey, microorganism, 2,3-butanediol, operating conditions, fermentation.

2.3 Introduction

Dairy industry is one of the most important industries inside the agri-food sector around the world. For instance, in Canada in 2015, the dairy industry generated almost 0.17 billion CAD representing 16% of the total profits generated by the agri-food sector (Canadian Dairy Information Center (CDIC) 2017a). Therefore, the dairy industry not only has an important economic impact but creates thousands of direct and indirect jobs, representing local and regional economic benefits (Castañeda Martínez et al. 2009). For example, in 2015 in Canada and Quebec (a province of Canada), the number of people employed in the dairy industry was close to 23000 and 8900, respectively (CDIC 2017b). However, the dairy industry also generates detrimental compounds for the environment such as: (1) greenhouses gases (GHG); mainly methane (CH_4) and carbon dioxide (CO_2) (FAO 2010); (2) liquids (wastewater and whey); around 11000 million m^3 of wastewater/year are generated in the world containing high organic loads (i.e. between 30 and 50 g of biochemical oxygen demand (BOD)/L water) (Mukhopadhyay et al. 2003; Tikariha and Sahu 2014), which could perturb the aquatic life; and (3) solids (manure, a cow produces an average of 62 kg of manure per day (feces and urine)) (Statistic-Canada 2006).

The dairy industry generates effluents when milk is processed. One of the most important products is cheese. One kg of manufactured cheese generates 9 L of whey, the main effluent of dairy industry (Guimaraes et al. 2008; Pintado et al. 2001). This must be processed to avoid the deterioration of the ecosystem. For this purpose, whey can be treated by coagulation-flocculation (Prazeres et al. 2012). It can also be upgraded by physicochemical treatments like membranes in order to get proteins (de Wit 2001; Madureira et al. 2007) or by biological treatments into biogas (hydrogen (H_2)), lactic acid, and alcohols such as 2,3-butanediol (2,3-BD) (Antonopoulou et al. 2008; Ghaly et al. 2003; Guimarães et al. 2010; Panesar et al. 2007; Parra Huertas 2009; Ukpai and Nnabuchi 2012).

2.4 Current status of the dairy industry

Milk for human consumption can be produced from cows, goats, etc. (Claeys et al. 2014; FAO-FAOSTAT 2017c). The worldwide production of milk was around 802 million tons in 2014, around 81% of the global production was cow milk (FAO-FAOSTAT 2017c).

Milk transformation produces a great variety of foods such as butter, yogurt or cheese. Cheese is one of the most consumed products (Muehlhoff et al. 2013). For example, in Canada, more than 1000 kinds of cheese are produced which include cheddar, cottage cheese and mozzarella (CDIC 2017c).

2.4.1 Current status in the world

In 2014, the worldwide production of cow milk was around 656 million tons. Among continents and countries, Europe and United States were respectively the largest global producers of cow milk, 33.1 and 14.3%, respectively (CDIC 2017d; FAO-FAOSTAT 2017c).

In the case of cheese, its worldwide production was 22.6 million tons of cheese produced in 2014 (FAO-FAOSTAT 2017d). Once again, Europe and United States were the largest producers of cheese in the world (52.4 and 24.7% of global cheese production respectively).

Milk and cheese production is an important part of the agro-food industry. Both products present an important role from an economic point of view for every country. For instance, in case of cheese, France exported around 33% of its cheese production (Vlahović et al. 2014). Hence, this fact in addition to the number of employees needed to process milk and manufacture cheese makes the dairy industry an important worldwide economic support (Hirsch and Hartmann 2014; Prakash 2015).

2.4.2 Current status in Canada and Québec

In 2015, the Canadian milk production was near 1.3% of world milk production, being the 5th biggest producer of cow milk in the American continent (CDIC 2017e; FAO-FAOSTAT 2017c). Quebec and Ontario were the main producers of milk (36.7 and 32.9% of the milk produced in Canada, respectively) (CDIC 2017e).

In the case of cheese, Canada produced near 1.9% of the world cheese production in 2015, this being the 3rd biggest producer of cheese in the American continent (FAO-FAOSTAT 2017d). Quebec and Ontario were the biggest producers (50.5 and 27.8%, respectively) of Canadian cheese (CDIC 2017f).

Hence, the dairy industry has a huge economic impact in Canada. For instance, in the particular case of Quebec, the dairy industry represented 38.2% of the number of employees in Canada (23322 in total) in 2015 (CDIC 2017b).

2.4.3 Environmental impacts of the dairy industry

On the other hand, milk production and its transformation cause important environmental impacts such as high consumption of energy and water, as well as emissions of gases, wastewater and solid waste (Capper et al. 2009; Saggar et al. 2004).

2.4.3.1 Gases

The main atmospheric pollutants emitted by the dairy industry are: ammonia (NH_3), nitrogen dioxide (NO_2), nitric oxide (NO) and GHG like CH_4, CO_2 and nitrous oxide (N_2O) (Saggar et al. 2004; United-Nations 2006). The gases are mainly due to animals metabolism (e.g., CH_4), the energy consumption necessary to operate barns and transport of raw materials and products, in addition to the transformation and preservation processes such as pasteurization, sterilization and refrigeration (Bertsch 2005; Ramirez et al. 2006). The dairy sector worldwide

emissions were near 1970 million tons of CO_2 equivalent in 2007 (4% of the total emissions of GHG) (FAO 2010). Milk production is responsible for 2.6% of global emissions of GHG in 2007 (FAO 2010). In the world, the average emission rates linked to the production of 1 kg of milk and 1 kg of cheese are 1 kg of CO_2 equivalent/kg milk and 8.8 kg CO_2 equivalent/kg cheese (Guignard et al. 2009). Therefore, the dairy industry is an important source of GHG.

2.4.3.2 Liquid effluents and consumption of water

The predominant environmental problem from the dairy industry is the large quantity of wastewater which is generated in two focal points: transformation processes and cleaning (CAR/PL 2002). The liquid effluents present high loads of organic matter from 2 to 10.2 g chemical oxygen demand (COD)/L; as well as phosphorus (P) and nitrogen (N) with loads up to 150 mg/L (phosphates (PO_4^{3-}) and pyrophosphates ($P_2O_7^{4-}$)) and 663 mg/L (organic nitrogen; ammonium (NH_4^+); nitrites (NO_2^-) and nitrates (NO_3^-)) (Cristian 2010; Demirel et al. 2005; Omil et al. 2003). In terms of the COD, its value can be higher than 60 g COD/L in streams from dairy products production (e.g., liquid stream from the cheese manufacturing, i.e. the whey) (Demirel et al. 2005; Omil et al. 2003).

Another point of consideration in understanding the environmental impact of the dairy industry is the rate of water consumption expressed as water footprint. For example, to produce 1 L of milk and 1 kg of cheese, 1 m^3 and 5 m^3 of fresh water are needed, respectively (Guignard et al. 2009; Mekonnen and Hoekstra 2010; Ridoutt and Pfister 2010).

2.4.3.3 Solid waste

Manure is the most important solid waste in the dairy industry. Solid cattle manure can contain up to 5 kg of N (NH_4^+, NH_3), 2.2 kg of P (phosphorus pentoxide (P_2O_5)), and 5 kg of K (potassium oxide (K_2O)) per metric ton of solid manure (Pennington et al., 2009; USEPA, 2012). Therefore, dry manure must be well managed because it can increase the salinity, pH and the concentrations of NH_4^+ and NO_3^- in soils and emissions of NH_3 gas by decomposition of manure. Ammonia can cause respiratory illness (CDC 2017c; Lehtomäki et al. 2007; Moral et al. 2005).

2.5 Whey

Whey is the liquid portion of milk which is obtained during the milk coagulation to produce cheese. Whey presents a high organic load (COD around 68 g/L) and thus it should be treated before being released to the environment as wastewater (Saddoud et al. 2007). Whey can also be valorized in high value added products such as nutritional food (e.g. as an additive for milk intended for children) and used in drugs manufacturing (Gunasekaran et al. 2007). In addition, whey can be used to obtain fermentative products such as alcohols (e.g., ethanol or 2,3-BD) or biogas (Chatzipaschali and Stamatis 2012; de Wit 2001; Dragone et al. 2009; Parra Huertas 2009; Perego et al. 2000). The valorization of whey depends on its composition which is a result of different factors including (1) the animal source of milk, (2) the kind of animal feed, (3) the stage of lactation, (4) the season of the year, (5) the acidity of whey and, (6) how the

cheese is processed (Madureira et al. 2007; Pescuma et al. 2015; Pintado et al. 2001). Figure 2.1 shows an overall scheme of the different processes used to valorize the whey produced by the dairy industry.

2.5.1 Whey generation

Figure 2.2 shows a simplified schematic representation of cheese and whey production from milk transformation. Raw milk is treated using a pasteurization process (a thermal process to reduce the population of pathogens). After the pasteurization stage, a culture of microorganisms (e.g., a strain of *Lactobacillus* or *Streptococcus*) is inoculated to the pasteurized milk to transform lactose into lactic acid (Miranda et al. 2009). Then, the coagulation process is performed. If the coagulation is carried out by rennet (complex of enzymes composed primarily of chymosin and pepsin), a sweet whey is generated (Miranda et al. 1989; Panesar et al. 2007); while if the coagulation process is performed by organic acids (e.g., acetic acid), an acid whey is obtained (Pintado et al. 2001). During the coagulation process, curd and whey are produced and thus have to be separated. The curd is then washed, cut, and pressed generating additional whey (Spalatelu 2012; Türkmenoglu 2006).

The cheese production has increased worldwide from $1.9x10^7$ in 2005, to $2.2x10^7$ tons in 2013. Hence, the whey generation around the world is estimated between $1.7x10^{11}$ L in 2005, to near $1.9x10^{11}$ L in 2013 (FAO-FAOSTAT 2017d; Smithers 2015). According to the CDIC (2017f), the production of cheese was $4.3x10^8$ kg in Canada in 2013 (i.e. $3.9x10^9$ L of whey).

2.5.2 Composition of whey

Whey is mainly composed of water which represents 93-95% (w/w) and contains around 50% of milk nutrients (Lievore et al. 2015). In 1 L of whey, there are between 60 and 70 g of solids (de Wit 2001; Panesar et al. 2007). The dry matter fraction is composed as follows: 66-77% (w/w) of lactose, 8-15% (w/w) of proteins and 7-15% (w/w) of minerals; and a lower portion of non-protein nitrogen (e.g., amino acids), vitamins (e.g., vitamins A, D and B5), trace elements (e.g., zinc) and proteins (e.g., lactoperoxidase) (Casper et al. 1998; Chegini and Taheri 2013; de Wit 2001; Ghaly et al. 2003; Panesar et al. 2007). The proteins of whey are albumins (up to 90% w/w), lactoferrin and immunoglobulins, which represent approximately 20% w/w of milk proteins (Keri Marshall 2004; Patel 2015). These proteins have antiviral, antimicrobial and antioxidative properties and thus whey proteins have become a very valued product in the food industry (Madureira et al. 2007; Patel 2015).

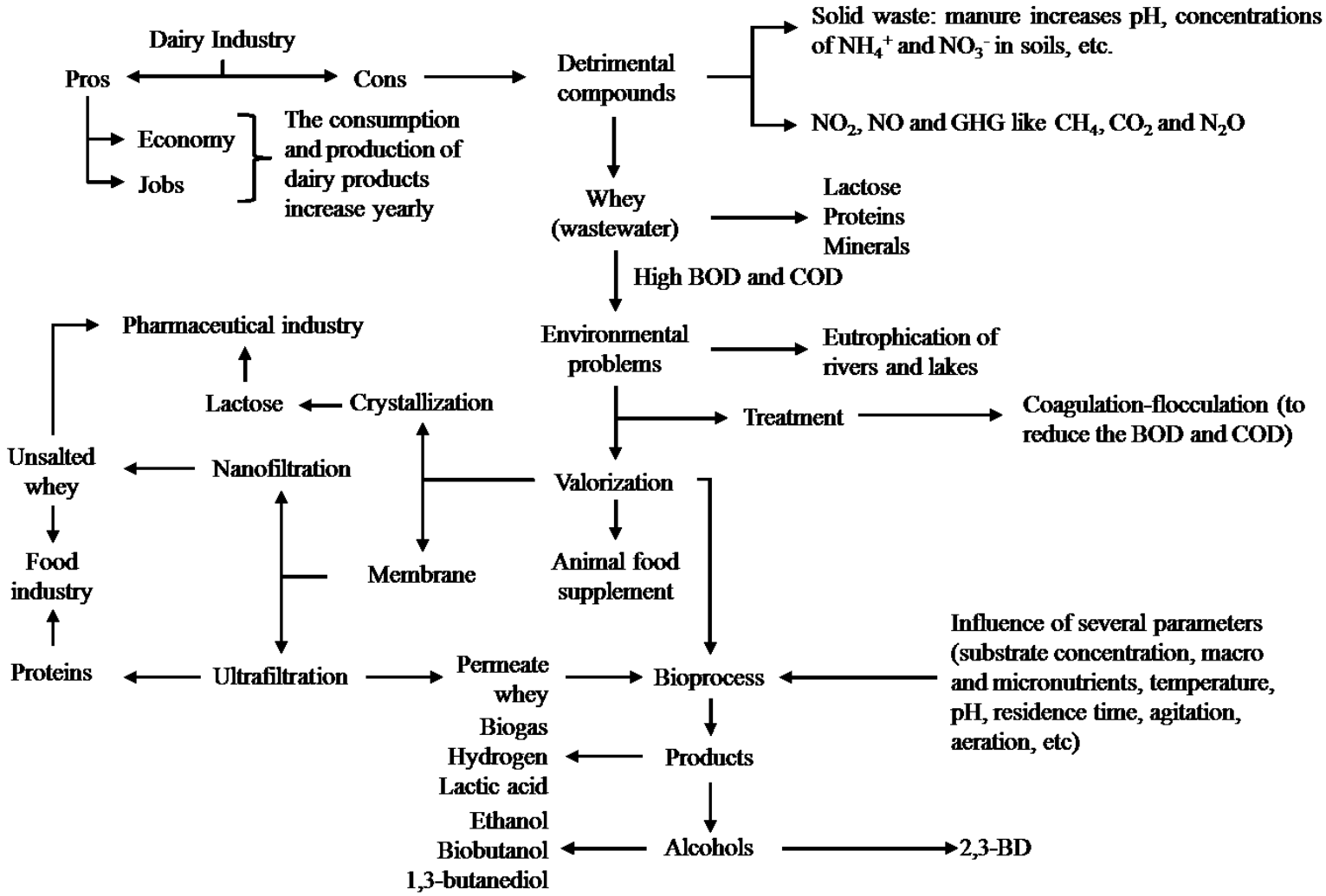


Figure 2.1: An overall scheme of the whey valorization processes

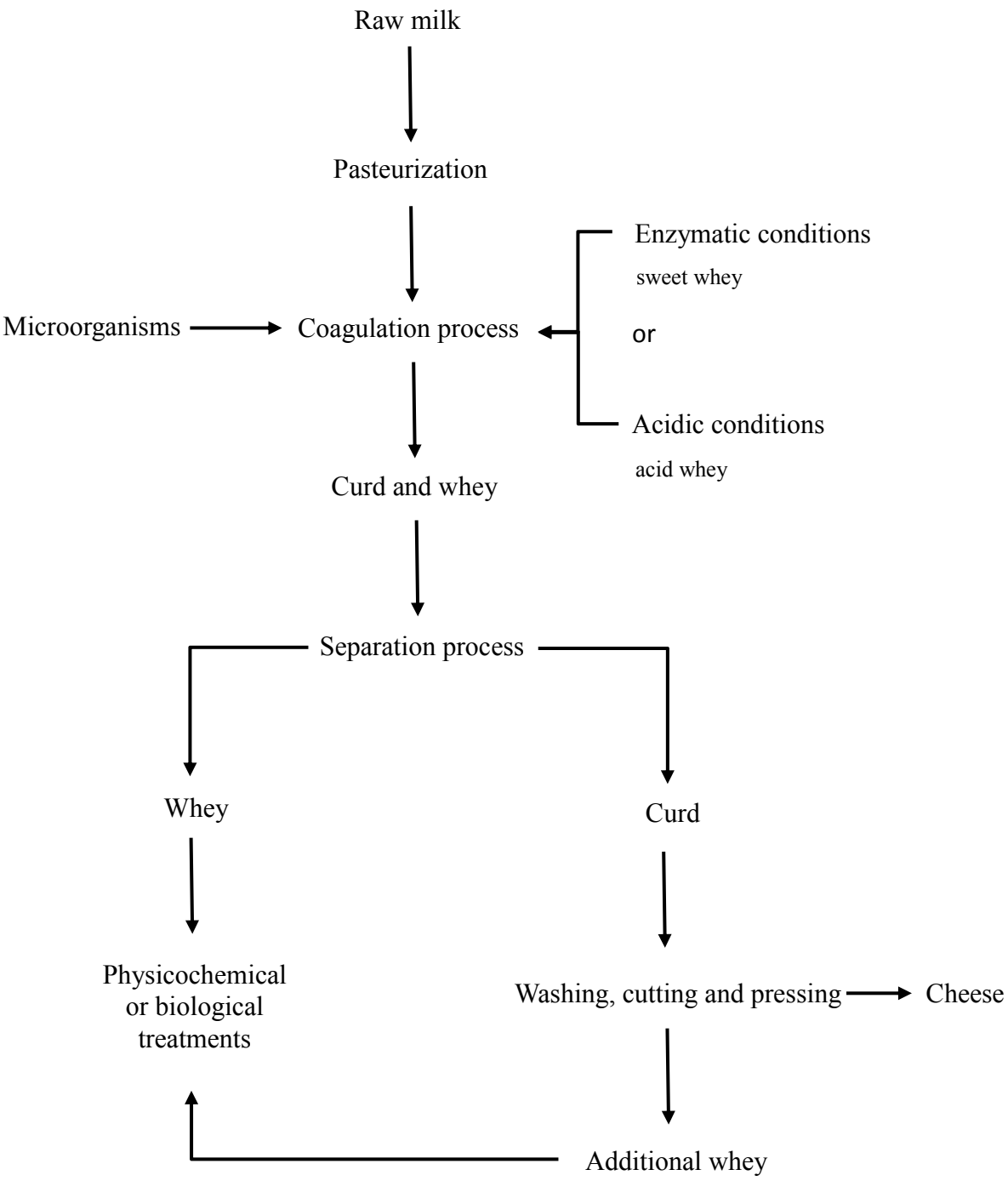


Figure 2.2: Whey production during cheese manufacturing

2.5.3 Acidity of whey

Milk coagulation is performed to generate curd and whey. The coagulation process determines the acidity and the composition of whey. Table 2.1 shows the differences between both types of whey depending on the generation process.

1) Sweet whey has a pH value around 6.5 and contains near 70 g/L of dry matter. The composition of dry matter is mainly formed by up to 77% (w/w) of lactose, up to 15% (w/w) of proteins, 15% (w/w) of minerals, up to 3% (w/w) of lactic acid besides trace elements, vitamins and minor proteins (Hernández-Rojas and Vélez-Ruiz 2014; Panesar et al. 2007; Pescuma et al. 2015; Tsakali et al. 2010).

2) Acid whey has a pH value, between 4.5 and 4.7 and contains nearly 70 g/L of dry matter mainly formed by up to 72% (w/w) of lactose, up to 15% (w/w) of proteins, up to 15% (w/w) of minerals and up to 13% (w/w) of lactic acid. When comparing sweet and acid wheys, it is possible to observe a smaller amount of lactose present in acid whey. This is due to a higher formation of lactic acid in the acid whey (Hernández-Rojas and Vélez-Ruiz 2014; Panesar et al. 2007; Pescuma et al. 2015; Tsakali et al. 2010).

Table 2.1: Composition of sweet and acid wheys. Source: Hernández-Rojas and Vélez-Ruiz (2014); Panesar et al. (2007); Pescuma et al. (2015); Tsakali et al. (2010).

Components	**Sweet whey (% (w/w))**	**Acid whey (% (w/w))**
	Enzymatic production	Acid production
Water	93 – 94	93 – 95
Dry matter	6.0 – 7.0	5.0 – 7.0
Lactose*	70 – 77	66 – 72
Proteins*	8 – 15	8 – 15
Minerals*	7 – 15	7 – 15
Lactic acid*	Up to 3	10 – 13

*Data based on the percentage (w/w) of the dry matter

2.5.4 Whey as an environmental problem

Whey is the most polluting substance issued from the manufacture of cheese (Prazeres et al. 2012). It has been estimated that only 50% of the whey is valorized into different products used in the food and chemical industries (Baldasso et al. 2011; Koutinas et al. 2014; Panesar et al. 2007). When whey is released as a wastewater, it can cause environmental problems. In order to be able to discharge the effluent in the environment, whey must be treated, for

example, by an ozonation treatment with hydrogen peroxide (Martins and Quinta-Ferreira 2010). This allows lactose and the rest of the organic loads of the whey to be degraded into CO_2 and water (H_2O) thus decreasing its negative environmental impact.

2.5.5 Processes applied to treat whey

Whey can directly be used as an animal food supplement since it is a source of proteins and minerals, or as a fertilizer without treatment (Audic et al. 2003; Baldasso et al. 2011; Schaafsma 2008). It is also possible to treat whey as a raw material in order to obtain added-value products (de Wit 2001; Karadag et al. 2015).

2.5.5.1 Physical and physicochemical processes

Whey can be treated by coagulation-flocculation using chemical compounds, such as iron (III) chloride ($FeCl_3$) or iron (II) sulfate ($FeSO_4$), to decrease the COD present in the whey. However, it is also possible to use membranes to obtain products (e.g., proteins) from whey (Canli 2005). Whey protein concentrates (WPC) are used in food such as emulsifier (Parra Huertas 2009; Rebouillat and Ortega-Requena 2015; Walzem et al. 2002). Whey possesses fat and casein particles in suspension which can be removed using a microfiltration process to prevent the obstruction of the ultrafiltration membranes (Das et al. 2015). The streams after recovering WPC by ultrafiltration is named permeate whey and contains a lactose concentration higher than 80 g/L of permeate (Becerra et al. 2015; de Wit 2001; FEDNA 2017; INTI 2017; USDairy 2017). Nanofiltration is also used to remove salts from whey. The non-salts whey can be used as follows: (1) in the food industry because it has a low salt content and thus prevents illnesses, such as hypertension; (2) in the pharmaceutical industry; and (3) in the chemical industry to make sugar-cellulose fibers (de Wit 2001; Minhalma et al. 2007; Rebouillat and Ortega-Requena 2015). The demineralization process can also be used to manufacture additives for milk intended for children (Parra Huertas 2009). The removal of lactose from whey, by crystallization or by using hollow fiber membranes, can be used in the pharmaceutical industry to make tablets for people unable to digest lactose; or lactose can be hydrolyzed to produce glucose and galactose which could be valorized by chemical or biological processes (Audic et al. 2003; Das et al. 2015; de Wit 2001).

2.5.5.2 Biological processes

Whey can be fermented to produce biogas, H_2, lactic acid, and different types of alcohols, such as ethanol, butanol and 2,3-BD (Roncal et al. 2010; Roncal et al. 2009).

1. Biogas

Biogas can be produced by anaerobic digestion of organic compounds (García et al. 2012). In this regard, whey can be used as feedstock because it contains lactose which could be fermented by microorganisms (e.g., *Methanobacterium*) under strict anaerobic conditions to

produce a biogas containing 50-70% (v/v) of CH_4, 30-40% (v/v) of CO_2 and 1-10% (v/v) of H_2 (COITAVC 2011; Ukpai and Nnabuchi 2012). The biogas yield depends mainly on the temperature and pH. If bacteria are mesophilic, the optimal temperature is ranged between 30 and 40ºC, while if bacteria are thermophilic, the temperature is ranged between 50 and 60ºC (Gerardi 2003; Khanal 2008). In the case of the pH, methanogenic bacteria produce biogas when the pH value is close to neutral (Ertem 2011). Whey as a culture medium (50 mL in serum bottles) produced around 23 L CH_4/L whey at 35ºC in 68 days (Ergüder et al. 2001). In Quebec (Canada), around 200000 m^3 of biogas/year are produced using more than 5 million of liters of whey (Chatzipaschali and Stamatis 2012).

2. Hydrogen

Traditionally, H_2 is derived from fuel processing (e.g., hydrocarbon reforming or pyrolysis); or from water (e.g., hydrolysis) but H_2 can also be obtained by anaerobic fermentation. Its production has been widely studied as an energy source in order to substitute fossil fuels due its higher heating value (HHV) of 142 kJ/g (Blanco Londoño and Rodríguez Chaparro 2012; Holladay et al. 2009; Pescuma et al. 2015; Sinha and Pandey 2011; USDE 2017). Nowadays, only 1% of worldwide production of H_2 is produced by anaerobic fermentation using microorganisms such as *Clostridia* (Ferchichi et al. 2005; Venetsaneas et al. 2009; Wu et al. 2008). For a day of production, 25 L of H_2 per L of whey were produced by means of fermentation in a 2-stage process (1 bar and 22ºC). A yield of 2.8 moles of H_2/mol of lactose was attained using granular sludge (an aggregate of microorganisms) (Cota-Navarro et al. 2011).

3. Lactic acid

Lactic acid has several applications in food, pharmaceutical, and chemical industries to give flavor (to increase the sweetness) and texture of products, and as a precursor of biodegradable polymers (Narayanan et al. 2004; Pescuma et al. 2008; Vijayakumar et al. 2008). Currently, near 90% of lactic acid produced around the world comes from the fermentation of saccharides by lactic acid bacteria (LAB) (Pescuma et al. 2015). These bacteria can use whey and thereafter hydrolyze the lactose in order to produce glucose and galactose (Black 2012; Pescuma et al. 2015). Glucose can be transformed into pyruvic acid by means of glycolysis, and the pyruvic acid can be fermented by lactic acid bacteria (e.g., *Lactobacillus*) into lactic acid (Hugenholtz and Kleerebezem 1999). Therefore, whey is a potential feedstock for fermentation processes due to its high content of lactose. For instance, up to 95% w/v of lactose from the whey can be transformed into lactic acid by *Lactobacillus casei* at 37ºC, pH 6.5 and 100 rpm, producing 0.84 g lactic acid/g of lactose for a reaction time of 36h (Panesar et al. 2010). The yield and concentration of lactic acid can be higher using a different species of *Lactobacillus*. A yield of 0.98 g lactic acid/g lactose was obtained for a reaction time of around 24h at 45ºC and pH 5.0 with *L. bulgaricus* NRRL B-548 (Venkatesh et al. 1993), whereas, Burgos-Rubio et al. (2000) found a lactic acid yield near 1.2 g lactic acid/g lactose for a reaction time of around 10h at 42ºC and pH 5.6 using the same bacteria (Burgos-Rubio et al. 2000).

4. Alcohols

Whey can be transformed into alcohols such as ethanol, butanol, 1,3-propanediol, and 2,3-BD (Becerra et al. 2015; Petrov and Petrova 2009; Wu et al. 2008).

The production of alcohols from whey is based on glucose fermentation; glucose is obtained from enzymatic hydrolysis (performed by enzymes, essentially β-galactosidases, at a temperature between 35 and 50ºC and a pH close to 7) or by chemical hydrolysis of lactose (using an acid, such as sulfuric acid, at a pH lower than 1.5 and a temperature higher than 150ºC) (Carrascal et al. 2010; Chatzipaschali and Stamatis 2012; Jurado et al. 2002; Khajavi et al. 2006; Neri et al. 2008; Oomori et al. 2004; Pereira-Rodríguez et al. 2012; Vieira et al. 2013). The alcoholic fermentation of whey can be economically competitive if concentrated whey is used (higher than 50 kg of lactose/m^3 of whey) (Guimarães et al. 2010; Prazeres et al. 2012). Alcohols are interesting for petrochemical and pharmaceutical industries because they can be transformed into other intermediate or final products such as ethylene, butylamine, poly(trimethylene terephthalate), or methyl ethyl ketone (Roncal et al. 2010; Roncal et al. 2009).

A. Ethanol

Lactose can be directly fermented by microorganisms and therefore whey is an excellent option as feedstock to produce ethanol (Padín González and Díaz Fernández 2009). The transformation of lactose into ethanol using microorganisms like *Kluyveromyces marxianus* UFV-3 can produce up to 0.535 g ethanol/g lactose (the theoretical value is 0.538 g ethanol/g lactose), i.e. a yield of 99% (g/g) under hypoxic conditions (injecting N_2) at 30ºC (pH and reaction time not specified) (Silveira et al. 2005).

B. Biobutanol

Biobutanol encompasses 4 different isomers; iso- and n-butanol are dominantly produced. The other isomers are *sec*- and *tert*-butanol (Becerra et al. 2015). Biobutanol has been traditionally produced by acetone-butanol-ethanol (ABE) fermentation (Green 2011). The mass ratio of ABE being 3:6:1 where butanol is the main product (Khanal 2008; Lee et al. 2012). This process is carried out in 2 steps: (1) acidogenesis, which produces acetic and butyric acids and (2) solventogenesis, which produces acetone, butanol and ethanol. Diverse types of saccharides (e.g., lactose) have been fermented using a *Clostridium* strain (Al-Shorgani et al. 2013; Becerra et al. 2015). In this case, using *Clostridium acetobutylicum*, the fermentation process yields 0.44 g ABE/g lactose after 470 h of fermentation at 35ºC, pH 5, and anaerobic conditions (Qureshi and Maddox 2005). However, a yield of 0.26 g butanol/g lactose using whey at 37ºC, pH 4.7 with a mean residence time of around 2h was obtained in a continuous fermentor using *Clostridium acetobutylicum* DSM792 (Raganati et al. 2013). Butanol possesses a HHV of 36.1 kJ/g, which is higher than the HHV of ethanol (29.8 kJ/g) and can be

used as a biofuel (Cleveland and Morris 2013; USDE 2017). Biobutanol is also the precursor of paints and products, such as butyl acrylate (Green 2011).

C. 1,3-propanediol

The diol, 1,3-propanediol (1,3-PD), is generally produced by microorganisms (e.g., *Clostridia*) which use glycerol as a carbon source, because no microorganism is able to ferment glucose into 1,3-PD in a natural way (Biebl et al. 1999; Patel et al. 2006). Therefore, 1,3-PD production from glucose is performed by genetically modified microorganisms e.g. *Escherichia coli* (Roncal et al. 2009). Here, glucose is transformed into glycerol (Saxena et al. 2009; Zeng and Biebl 2002). Using glycerol as a substrate, the yield of 1,3-PD can reach 0.90 g 1,3-PD/g glycerol by a modified strain of E. coli at pH 7 and with a combination of aerobic (0 to 10h) and anaerobic (10 to 40h) conditions at 30 and 42ºC, respectively (Tang et al. 2009). Using glucose as a carbon source, the yield of 1,3-PD reached 0.40 g 1,3-PD/g glucose with an engineered strain of E. coli at 37°C after 60h (pH not defined) (Liang et al. 2011).

Therefore, lactose contained in the whey may be used as feedstock to produce 1,3-PD if lactose is previously hydrolyzed into its 2 monosaccharides, glucose and galactose. 1,3-PD is employed to obtain polymers, such as polyesters, polyethers and polyurethanes (Xiu and Zeng 2008).

D. 2,3-butanediol

The diol, 2,3-BD, can be obtained from petroleum by chemical processes, which present "hard" operating conditions (high temperature, superior to 150ºC, low pH, inferior to 1.5 and expensive catalyzers) (Guimarães et al. 2010; Jiang et al. 2014). For this reason, the 2,3-BD could be produced by fermentation, a green technology with "softer" conditions of temperature and pH. Saccharides like arabinose, glucose, inulin, lactose, maltodextrin, sucrose could be used as substrates (Celińska and Grajek 2009; Garg and Jain 1995; Xiao et al. 2012). Whey can also be used in order to produce 2,3-BD. But, whey has to be previously hydrolyzed into glucose and galactose by microorganisms like *Klebsiella* (Afschar et al. 1993; Casper et al. 1998; Ghaly et al. 2003; Shin et al. 2012; Wong et al. 2014; Xiu and Zeng 2008).

The diol, 2,3-BD, can be used as additive for fuels due to its HHV (27.2 kJ/g), which is superior to the HHV of alcohol like methanol (22.7 kJ/g) (Ge et al. 2011; USDE 2017). 2,3-BD is also the precursor of (1) MEK, which is used as a fuel additive, (2) 1,3-butanediol, which is used to manufacture polyesters and plasticizers, (3) 2-butene, which is used in butadiene production (Roncal et al. 2010). The biological process to transform 2,3-BD into diverse products has recently attracted the interest of industries since bioprocesses reduce the dependence on petroleum and decrease the CO_2 emissions (Savakis et al. 2013).

2.6 Metabolic pathway from lactose to 2,3-BD

Figure 2.3 presents a simplified diagram of the metabolic pathway from lactose to 2,3-BD which starts with the enzymatic hydrolysis of lactose (Spalatelu 2012). Lactose is hydrolyzed using the β-galactosidase enzyme encoded in the *lacZ* gene of the lactose operon, which is formed by the genes *lacZ*, *lacY* and *lacA* (Alpers and Tomkins 1966). *LacY* encodes the enzyme galactoside permease that facilitates the transport of lactose into the cell, while *lacA* encodes the enzyme thiogalactoside transacetylase where its role is still not fully understood (Esmaeili et al. 2015).

Thus, the metabolism of lactose to 2,3-BD starts when the galactoside permease enzyme transports the lactose into the bacteria and the β-galactosidase enzyme breaks down 1 molecule of lactose and produces 2 molecules of monosaccharides (1 molecule of glucose and 1 molecule of galactose) (Alpers and Tomkins 1966; Black 2012). Once glucose has been generated, glycolysis begins. In this way, glucose is transformed into pyruvic acid which is transformed into 2,3-BD (Xu et al. 2014). The enzyme α-acetolactate synthase (ALS) transforms pyruvic acid into α-acetolactate. This step is improved at approximately pH 6.0 (Ji et al. 2011). Then, α-acetolactate is fermented to acetoin by the enzyme α-acetolactate decarboxylase (ALDC). Finally, acetoin is transformed into 2,3-BD by means of the enzyme 2,3-BD dehydrogenase (BDH) (Ji et al. 2011; Magee and Kosaric 1987).

2.6.1 Effect of microorganisms and operating conditions on 2,3-BD production

Fermentation of saccharides to produce 2,3-BD is influenced by different operating conditions (Table 2.2), such as the type of microorganisms, substrate concentration, macro and micronutrients, temperature, pH, residence time, agitation, aeration, etc. (Celińska and Grajek 2009; Hespell 1996; Nakashimada et al. 2000).

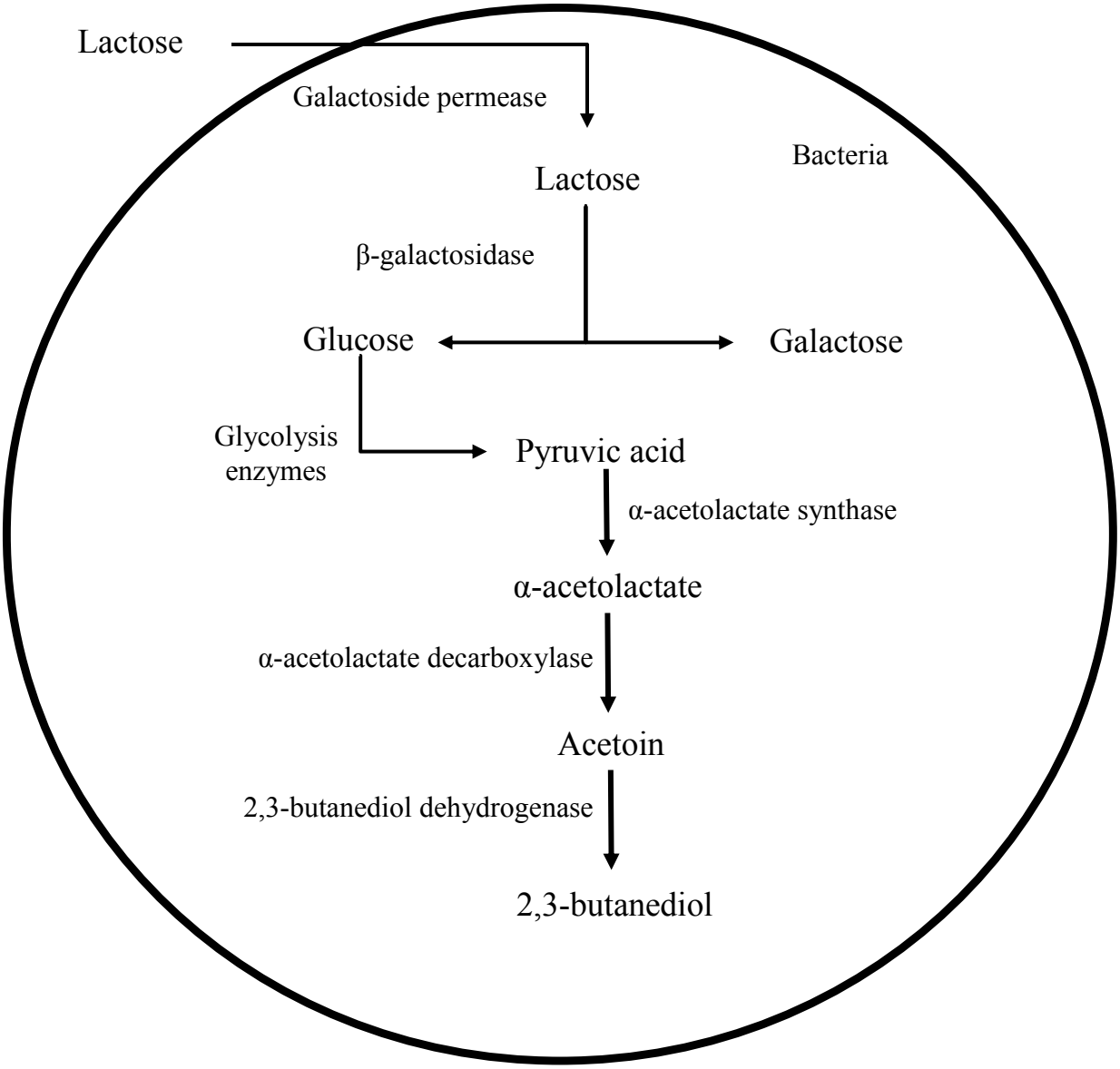


Figure 2.3: Representation of the metabolic pathway from lactose to 2,3-butanediol. Source: Alpers and Tomkins (1966) and Xu et al. (2014).

Table 2.2: Operating conditions using different types of microorganisms and their influence on the maximum 2,3-butandediol yield.

Variable operating conditions	Microorganism inoculum	Substrate and initial concentration	T (ºC)	pH	Aeration (vvm)	Agitation (rpm)	Reactor operation mode	Working volume (L)	Nutrient	Time (h)	Maximum 2,3- BD yield (g 2,3-BD/g substrate)	References
Substrate	*K. pneumoniae* NRRL B199 (10% (v/v))	Sucrose (95 g/L)	37	5.5	0.8	230	Batch	2.0 (reactor volume)	No	34.5	0.49	Silveira et al. (1998)
	K. sp. XMR21 (2% (v/v))	Sucrose (50 - 100 g/L)	35	6.0	-	150	Batch	0.05	No	48	0.43	Xin et al. (2016)
	K. oxytoca KMS005 (0.033 g/L)	Maltodextrin (200 g/L)	37	6.0	1.0	400	Batch	1.0	No	72	0.37*	Chan et al. (2016)
	E. aerogenes NCIMB 10102 (0.25 g/L)	Glucose (20 g/L)	39	6.0	-	150	Batch	1.5	No	250	0.40	Perego et al. (2000)
	E. aerogenes NCIMB 10102 (0.25 g/L)	Pre-hydrolyzed whey (20 g/L)	39	6.0	-	150	Batch	1.5	No	199	0.43	Perego et al. (2000)
	E. cloacae SG1 (2% (v/v))	Glucose (30 g/L)	30	6.5	-	200	Batch	-	No	48	0.48	Hazeena et al. (2016)
	E. cloacae SDM09 (5% (v/v))	Glucose:xylose (3:1 (w/w), N.D)	30	7.0	1.0	500	Fed-batch	1.0 (reactor volume)	No	50	0.50	Li et al. (2015)

Table 2.2: Operating conditions using different types of microorganisms and their influence on the maximum 2,3-BD yield (continued)

Variable operating conditions	Microorganism inoculum	Substrate and initial concentration	T (ºC)	pH	Aeration (vvm)	Agitation (rpm)	Reactor operation mode	Working volume (L)	Nutrient	Time (h)	Maximum 2,3- BD yield (g 2,3-BD/g substrate)	References
Compounds stimulating of the process	*K. oxytoca* PTCC 1402 (8 g/L)	Glucose (6 g/L)	37	6.0	-	150	Batch	0.1	Acetic acid (0.5 g/L)	N.D.	0.47	Anvari and Motlagh (2011)
	K. pneumoniae HR521 LDH (2% (v/v))	Xylose (90 g/L)	37	6.5	-	200	Batch	0.1	Acetic acid (4.5 g/L)	36	0.39*	Wang et al. (2016)
	K. pneumoniae HR521 LDH (5% (v/v))	Xylose (90 g/L)	37	6.5	2.5	300	Batch	4.0	Acetic acid (2.5 g/L) Yeast extract (35.2 g/L)	36	0.47	Wang et al. (2016)
	P. polymyxa DSM 365 (10% (v/v))	Sucrose (N.D.)	37	6.0	0.2	500	Fed-batch	2.0 (reactor volume)	Yeast extract (60 g/L)	54	2.06 g/(L·h)***	Häβler et al. (2012)
	Klebsiella sp. Zmd30 (2% (v/v))	Glucose (160 g/L)	30	N.D.	-	200	Batch	0.1	Urea (2.0 g/L)	N.D.	0.35*	Wong et al. (2012)
	K. oxytoca ME-303 (5% (v/v))	Glucose-Xylose (2:1 (w/w), 60 g/L)	37	6.5	-	200	Batch	2.0	Urea (26.4 g/L)	44	0.39	Ji et al. (2009b)
	P. polymyxa ZJ-9 (8% (v/v))	Inulin (77 g/L)	30	6.0	-	240 (0-24 h) 120 (24-48 h)	Batch	3.0	K_2HPO_4 (3.1 g/L)	48	0.48*	Gao et al. (2010)
	K. pneumoniae CICC 10011 (5% (v/v))	Glucose (135 g/L)	37	6.0	-	N.D.	Batch	0.1	$(NH_4)_2HPO_4$ (24 g/L) $MgSO_4.7H_2O$ (0.9 g/L)	55	0.39*	Jiayang et al. (2006)
	Klebsiella sp. Zmd30 (2% (v/v))	Glucose (160 g/L)	30	N.D.	-	200	Batch	0.1	$MnCl_2.4H_2O$, $CoCl_2.6H_2O$ (0.7 and 5.7 mg/L respectively)	N.D.	1.15 g/(L·h)***	Wong et al. (2012)

Table 2.2: Operating conditions using different types of microorganisms and their influence on the maximum 2,3-BD yield (continued)

Variable operating conditions	Microorganism inoculum	Substrate and initial concentration	T (ºC)	pH	Aeration (vvm)	Agitation (rpm)	Reactor operation mode	Working volume (L)	Nutrient	Time (h)	Maximum 2,3- BD yield (g 2,3-BD/g substrate)	References
Temperature	*B. amyloliquefaciens* B10-127 (4% (v/v))	Glucose (150 g/L)	37	6.5	-	160	Batch	0.05	No	82	0.39	Yang et al. (2011)
	B. subtilis 168 (2% (v/v))	Glucose (10 g/L)	37-46	6.5	-	100	Batch	0.1	No	N.D.	0.35*	Fu et al. (2016)
	B. licheniformis NCIMB 8059 (0.5 g/L)	Glucose (20 g/L)	37	6.0	-	150	Batch	1.5	No	N.D.	0.11	Perego et al. (2003)
pH	*E. cloacae* SG1 (2% (v/v))	Glucose (30 g/L)	30	6.5	-	200	Batch	-	No	48	0.47*	Hazeena et al. (2016)
	E. cloacae NRRL B-23289 (4% (v/v))	Arabinose (50 g/L)	30	5.1	-	200	Batch	0.05	No	32	0.40	Saha and Bothast (1999)
	E. coli BL21 (10% (v/v))	Glucose (N.D.)	N.D.	8.0	1.0	400	Batch	0.8	No	18	0.43*	Xu et al. (2014)
	E. coli YJ3 (10% (v/v))	Glucose (80 g/L)	37	6.5	1.5	400	Batch	2.0	No	18	0.38*	Tong et al. (2016)
Fermentation time	*K. oxytoca* ME-XJ-8 (5% (v/v))	Glucose (200 g/L)	37	6.5	1.0	200	Batch	2.0	No	60	0.48	Ji et al. (2010)
	K. oxytoca ME-UD-3 (5% (v/v))	Glucose (200 g/L)	37	6.5	1.0	200	Batch	2.0	No	60	0.45	Ji et al. (2010)
	E. coli JM109 (2% (v/v))	Glucose (40 g/L)	30	7.5	-	160 spm	Batch	0.1	No	24	0.27	Ui et al. (1997)
	K. pneumonia G31 (10% (v/v))	Glycerol (N.D.)	37	5.2	0.44	200	Fed-batch	N.D.	No	234	0.38*	Petrov and Petrova (2009)

Table 2.2: Operating conditions using different types of microorganisms and their influence on the maximum 2,3-BD yield (continued)

Variable operating conditions	Microorganism inoculum	Substrate and initial concentration	T (ºC)	pH	Aeration (vvm)	Agitation (rpm)	Reactor operation mode	Working volume (L)	Nutrient	Time (h)	Maximum 2,3- BD yield (g 2,3-BD/g substrate)	References
Agitation	*K. oxytoca* KMS005 (0.033 g/L)	Maltodextrin (100 g/L)	37	6.0	1.0	300	Batch	1.0	No	48	0.37*	Chan et al. (2016)
	E. coli BL21 (10% (v/v))	Glucose (N.D.)	N.D.	7.0	1.0	400	Batch	0.8	No	17	0.44*	Xu et al. (2014)
	K. oxytoca ME-UD-3 (5% (v/v))	Glucose (200 g/L)	37	6.5	1.0	300 (0-15h) 200 (15-68h)	Batch	2.0	No	56	0.48	Ji et al. (2009a)
	E. cloacae TERI BD18 (2% (v/v))	Glucose (N.D.)	37	7.5	-	200 (0-10h) 150 (10-80h)	Fed-batch	100.0	No	50	0.48**	Priya et al. (2016)
	E. cloacae NRRL B-23289 (4% (v/v))	Arabinose (50 g/L)	30	5.0	-	100	Batch	0.05	No	144	0.40	Saha and Bothast (1999)
	E.coli YJ3 (10% (v/v))	Glucose (80 g/L)	37	6.5	1.5	400	Batch	2.0	No	18	0.38	Tong et al. (2016)
Aeration	*K. oxytoca* KMS005 (0.033 g/L)	Maltodextrin (100 g/L)	37	6.0	1.2	200	Batch	1.0	No	48	0.35	Chan et al. (2016)
	B. subtilis 168 (2% (v/v))	Glucose (105 g/L)	37	6.5	0.02	300	Batch	2.3	No	86	0.49	Fu et al. (2016)
	S. marcescens G12 (5% (v/v))	Glucose (N.D.)	35	6.0	1.0 – 2.5 (0-30 h) 2.5 – 1.0 (30-70h)	400	Fed-batch	5.0 (reactor volume)	No	68	0.49*	Shi et al. (2014)

N.D. = Non defined. * Calculated using the experimental data provided by the authors. ** Yield provided by the authors. ***Productivity

2.6.1.1 Microorganism

The production of 2,3-BD from different carbon sources like glucose, lactose, sucrose, starch, etc. can be performed by fermentation in the presence of bacteria, such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Paenibacillus polymyxa*, *Serratia marcescens*, etc. (Fu et al. 2016; Mazumdar et al. 2013; Nakashimada et al. 1998; Perego et al. 2000; Perego et al. 2003; Petrov and Petrova 2009; Priya et al. 2016; Shi et al. 2014; Xin et al. 2016; Yang et al. 2011). Table 2.3 presents several characteristics and particularities of these bacteria.

Table 2.3: Characteristics and particularities of some bacteria producers of 2,3 butanediol

Genus	Strain	Characteristics	Particularities	Reference
Enterobacter	*E. cloacae* *E. aerogenes*	Gram-negative bacteria Facultative anaerobic bacteria Rod-shaped bacteria Non-spore-forming bacteria	Human pathogen High ability to keep the damage and invasion of tissue host	Davin-Regli 2015; Santo Pereira et al. 2016
Klebsiella	*K. pneumoniae** *K. oxytoca*	Gram-negative bacteria Facultative anaerobic bacteria Rod-shaped bacteria Non-motile bacteria Non-spore-forming bacteria	Human pathogen High ability and speed to grow High resistance against penicillin* High resistance against host defenses due to its prominent polysaccharide capsule	PHAC 2017; Trivedi et al. 2015a, b
Bacillus	*B. amyloliquefaciens* *B. licheniformis* *B. subtilis*	Gram-positive bacteria Facultative anaerobic/aerobic bacteria Spore-forming bacteria Rod-shaped bacteria Motile bacteria	Generally regarded as safe (GRAS) Resistance to heat, chemical products and irradiation	Bhandari et al. 2013; de Clerck et al. 2004; Erem et al. 2009; Fu et al. 2016; Wulff et al. 2002; Yang et al. 2011

Natural producers of 2,3-BD like *E. cloacae*, *K. oxytoca* or *S. marcescens* possess the genes needed to produce the enzymatic machinery to be employed in the 2,3-BD metabolic pathway (Ji et al. 2011). These natural producers of 2,3-BD gave 2,3-BD yields close to 0.50 g 2,3-BD/g saccharide such as glucose (monosaccharide), sucrose (disaccharide) or inulin

(polysaccharide) (Gao et al. 2010; Shi et al. 2014; Silveira et al. 1998). For example, a 2,3-BD yield of 0.49 g 2,3-BD/g glucose (calculated using the data provided by the authors) at 35ºC, pH 6.0 and 400 rpm was obtained in the presence of *S. marcescens* G12 (5% (v/v) of inoculum) after 68 h of fermentation. The aeration rate varied between 1 and 2.5 vvm using a working volume of 5 L (fed-batch fermentor and substrate concentration undefined) (Shi et al. 2014). A 2,3-BD yield of 0.49 g 2,3-BD/g sucrose was obtained with *K. pneumoniae* NRRL B199 (10% (v/v) of inoculum) after 34.5 h of batch fermentation using 95 g/L of sucrose at 37ºC, pH 5.5, 0.8 vvm and 230 rpm in a fermentor of 2 L (Silveira et al. 1998). Using a response surface methodology, Gao et al (2010) found that *P. polymyxa* ZJ-9 (8% (v/v) of inoculum) gave a similar yield (0.48 g 2,3-BD/g inulin) in a batch reactor (3 L of working volume) after 48 h fermenting 77 g/L of inulin at 30ºC, pH 6.0 and 240 rpm (0-24 h) and 120 rpm (24-48 h). Interestingly, *E. aerogenes* and *E. cloacae* can also ferment agro industrial wastes such as lactose derived from whey or a mixture of glucose and xylose. For example, *E. aerogenes* NCIMB 10102 (0.25 g/L of inoculum) fermented 20 g/L of pre-hydrolyzed whey in a working volume of 1.5 L, which resulted to a yield of 0.43 g 2,3-BD/g pre-hydrolyzed whey at 39ºC, pH 6.0, 150 rpm and 199 h of batch fermentation (Perego et al. 2000). On the other hand, *E. cloacae* SDM09 (5% (v/v) of inoculum) provided a yield of 0.50 g 2,3-BD/g glucose:xylose (3:1 (w/w), mixture concentration undefined) (calculated using the data provided by the authors) in a 1 L of fed-batch fermentor at 30ºC, pH 7.0, 1 vvm and 500 rpm for 50 h (Li et al. 2015).

Klebsiella strains like *K. pneumoniae* and *K. oxytoca* are two of the most used microorganisms in the literature to produce 2,3-BD because they can generate high concentrations of 2,3-BD (up to 150 g 2,3-BD/L representing, for example, a 2,3-BD yield of 0.48 g 2,3-BD/g glucose) (Ma et al, 2009). They are also easy to cultivate and they grow relatively quickly (cell duplication time shorter than 1h) (Białkowska 2016; Black 2012). However, the main disadvantage of *Klebsiella* is its biosafety level 2, i.e., it is a human pathogen microorganism (Lu et al. 2012). Other promising 2,3-BD producers like *E. aerogenes*, *E. cloacae* and *S. marcescens* also possess biosafety level 2 (CDC 2017b; Tong et al. 2016). Therefore, the use of these bacteria for producing 2,3-BD at industrial scale is limited.

To overcome the biosecurity issue of *Enterobacter*, *Klebsiella* and *Serratia* strains, the 2,3-BD can be obtained by using genetically engineered *E. coli*. A wide number of *E. coli* strains, like *E. coli* W3110, K-12 or BL21, are harmless for humans with a biosafety level 1 (ABSA 2017; ATCC 2016; CDC 2017a; Fukiya et al. 2004; iGEM 2017; Jin et al. 2002; Xu et al. 2014). Nevertheless, *E. coli* does not have the genes *budB*, *budA* and *budC* which encode the enzymes α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC), and 2,3-butanediol dehydrogenase (BDH) respectively required to produce 2,3-BD (Shin et al. 2012; Xu et al. 2014). It is therefore necessary to add the 2,3-BD metabolic pathway into *E. coli* genome from other microorganisms like *P. polymyxa* or *E. cloacae* (Lara 2011; Portnoy et al. 2008; Xu et al. 2014). *E. coli* presents several advantages: (1) a wide knowledge of its genome, (2) an easy genetic manipulation to add metabolic pathways from other bacteria; and (3) a wide and available number of substrates for fermentation (like glucose, lactose, starch, etc.) to produce 2,3-BD (Tong et al. 2016; Wendisch et al. 2016). Moreover, it is also possible to block some metabolic pathways which produce end-products, such as acetate, ethanol, lactate and succinate, during the fermentation. The removal of these metabolic routes can affect the 2,3-BD yield produced by genetically modified *E. coli* in comparison to natural

producer bacteria (e.g. *E. cloacae*, *K. pneumoniae* or *S. marcescens*) (Mazumdar et al. 2013). A 2,3-BD yield of 0.44 g 2,3-BD/g glucose was obtained after 17 h at a pH of 7.0, 1 vvm and 400 rpm in a batch reactor (0.8 L of working volume; glucose concentration undefined) in the presence of *E. coli* BL21 (10% (v/v) of inoculum) (Xu et al. 2014). *E. coli* YJ3 (10% (v/v) of inoculum) gave a 2,3-BD yield of 0.38 g 2,3-BD/g glucose after 18 h, fermenting 80 g/L of glucose at 37ºC, pH 6.5, 1.5 vvm and 400 rpm in a batch reactor (2 L of working volume) (Tong et al. 2016). The modified strains of *E. coli* are potential 2,3-BD producers.

Furthermore, the production of specific 2,3-BD isomers ((S, S)-, meso- and (R, R)-2,3-BD) can also direct the selection of the bacteria. These isomers have different applications. For instance, R, R-2,3-BD is used in the production of antifreezes (Qi et al. 2014), while meso-2,3-BD is dehydrated to obtain 1,3-butadiene, involved in the polyesters synthesis (Qiu et al. 2016) and S, S-2,3-BD is dehydrogenated to synthesize butanedione (Białkowska 2016). For example, a specific 2,3-BD isomer like R, R-2,3-BD can be obtained with *E. coli* by cloning the gene encoding the R, R-2,3-BDH enzyme obtained from *B. licheniformis* (Jiang et al. 2014). In the presence of *Klebsiella* strains and *S. marcescens*, a mixture of 2,3-BD isomers can be produced, whereas the isomers (R, R)- and meso-2,3-BD can be formed at a purity of 98 and 99% (w/w) with *P. polymyxa* and *B. subtilis* respectively (Fu et al. 2016; Ji et al. 2011; Ji et al. 2015; Jiang et al. 2014).

The operating conditions influencing the 2,3-BD production like substrate concentration, macro and micronutrients, temperature, pH, residence time, agitation and aeration will be presented in the following subsections.

2.6.1.2 Influence of the substrate

The substrate can be a monosaccharide, a disaccharide or a polysaccharide like glucose, lactose and inulin, respectively. These saccharides can be transformed into 2,3-BD by bacteria and the price of 2,3-BD is strongly linked to the raw material (carbon substrate) cost (Celińska and Grajek 2009). The food industry residues like corncob molasses and whey are good sources of saccharides since they are cheap, available and renewable (Ji et al. 2011). Although, it could be necessary to perform a pretreatment at high temperature and in presence of acid in order to break the saccharide structure (Białkowska 2016). On the other hand, the substrate concentration is also an important factor on the 2,3-BD production. The substrate could cause osmotic stress in bacterial cells if its concentration is higher than the one inside the cell (Krämer 2010).

Each microorganism possesses a substrate concentration threshold and beyond this value, an inhibition of metabolism or growth could occur (Priya et al. 2016). For example, a range of sucrose concentration varying between 20 and 204 g/L was fermented in the presence of *K. pneumoniae* NRRL B199 (10% (v/v) of inoculum) at 37ºC, pH 5.5, 230 rpm and 0.8 vvm in a fermentor of 2 L (Silveira et al. 1998). In the range from 20 to 95 g/L of sucrose, the 2,3-BD yield increased by 48% (from 0.33 to 0.49 g 2,3-BD/g sucrose); whereas the fermentation time increased by 3 times (from 11.4 and 34.5 h fermenting 20 and 95 g/L, respectively). Nevertheless, when the sucrose concentration reached 204 g/L, the 2,3-BD yield and the fermentation time decreased by 16% and increased by 132% respectively (0.41 g 2,3-BD/g sucrose and 80 h) (Silveira et al. 1998). Xin et al. (2016) also fermented sucrose in a range

between 50 and 300 g/L in the presence of *Klebsiella* sp. XMR21 (2% (v/v) of inoculum) at 35ºC, pH 6.0 and 150 rpm in 0.05 L of culture medium during 48 h. The maximum 2,3-BD yield (0.43 g 2,3BD/g sucrose) was obtained using 50 and 100 g/L of sucrose. In the presence of 150 or 200 g/L of sucrose, the 2,3-BD yield dropped by 42% (0.25 g 2,3BD/g sucrose for both concentrations). Finally, the 2,3-BD yield continued falling down to 0.14 g 2,3BD/g sucrose at 300 g/L of sucrose.

The substrate threshold concentration might depend also on the substrates. For example, a range of maltodextrin (a polysaccharide) concentrations varying between 50 and 250 g/L, was fermented for 48 and 72 h respectively in the presence of *K. oxytoca* KMS005 (0.033 g/L of inoculum) at 37ºC, pH 6.0, 1 vvm and 400 rpm (Chan et al. 2016). For an increase of the maltodextrin concentration ranging from 50 to 200 g/L, the 2,3-BD yield increased up to 0.37 g 2,3-BD/g maltodextrin at 200 g/L of maltodextrin and decreased by 1.3 fold in the presence of 250 g/L of maltodextrin (calculated from the data provided by the authors).

On the other hand, certain bacterial strain can maximize the 2,3-BD yield at a low substrate concentration like 20 or 30 g/L. At a 30 g/L of glucose in the presence of *E. cloacae* SG1 (2% (v/v) of inoculum) at 30ºC (an initial pH of 6.5 and 200 rpm during 48 h), the 2,3-BD yield (0.48 g 2,3-BD/g glucose) increased by 27% compared to 60 g/L of glucose (Hazeena et al. 2016). In the presence of *E. aerogenes* NCIMB 10102 (0.25 g/L) a maximum 2,3-BD yield of 0.40 g 2,3-BD/g glucose was obtained by fermenting 20 g/L of glucose which increased by 18% compared to 100 g/L of glucose. The operating conditions were 39ºC, pH 6.0, 150 rpm and 1.5 L of fermentation medium (Perego et al. 2000).

2.6.1.3 Influence of the compounds stimulating of the process

The fermentation of saccharides into 2,3-BD can be enhanced by adding nutrients like a nitrogen source (for instance yeast extract (YE)), phosphate sources like potassium monohydrogen phosphate (K_2HPO_4) or ammonium hydrogen phosphate ($(NH_4)_2HPO_4$), metal ions like magnesium (Mg^{2+}), manganese (Mn^{2+}) and cobalt (Co^{2+}), etc. (Gao et al. 2010; Häßler et al. 2012; Nakashimada et al. 2000; Wong et al. 2012).

a) Nitrogen

Yeast extract is a nitrogen source and it also provides essential substances like vitamins and amino acids needed for the microorganism development (Garg and Jain 1995). Yeast extract contains methionine, which is not synthesized by bacteria like *P. polymyxa* and could increase the 2,3-BD yield (Li et al. 2013). In addition, methionine is an essential amino acid for microorganisms and the precursor of proteins (Garg and Jain 1995; Levine et al. 2000; SM 2017). Two concentrations of YE (5 and 60 g/L) were used in a fed-batch fermentor of 2 L (sucrose concentration undefined) in the presence of *P. polymyxa* DSM 365 (10% (v/v)) at 37ºC, pH 6.0, 500 rpm and 0.2 vvm for 54 h. The addition of 60 g/L of YE doubled the 2,3-BD productivity (up to 2.06 g 2,3-BD/(L·h)) in comparison with 5 g/L of YE (0.98 g 2,3-BD/(L·h)) (Häßler et al. 2012).

The price of YE being high, other nitrogen sources like urea were tested (Ji et al. 2011; Wong et al. 2012). For example, Wong et al. (2012) tested *Klebsiella* sp. Zmd30 (2% (v/v) of inoculum) with urea concentrations varying between 0 and 10 g/L at 30ºC and 200 rpm in 0.1 L of fermentation medium with 160 g/L of glucose. In the absence of urea, the 2,3-BD yield was practically null (0.02 g 2,3-BD/g glucose), while by adding 2 g/L of urea, the 2,3-BD yield reached 0.35 g 2,3-BD/g glucose. Furthermore, the 2,3-BD yield remained constant at 0.30 g 2,3-BD/g glucose with the urea concentrations varying from 4 to 10 g/L (Wong et al. 2012). However, the urea concentration needed to maximize the 2,3-BD yield could be higher than 2 g/L. For example, Ji et al. (2009b) reported a 2,3-BD yield of 0.39 g 2,3-BD/g glucose-xylose using *K. oxytoca* ME-303 (5% (v/v) of inoculum) in the presence of 26.4 g/L of urea (ranged from 10 to 30 g/L) at 37ºC, pH 6.5 and 200 rpm in 2 L of basal medium supplemented with 60 g/L of a mixture of glucose-xylose (ratio of 2:1 (w/w)) (Ji et al. 2009b). Hence, the urea can be considered as an alternative nitrogen source to YE in order to produce 2,3-BD.

b) Phosphate

Phosphates like $(NH_4)_2HPO_4$ and K_2HPO_4 improve the 2,3-BD yield because: (1) they affect the performance of enzymes (e.g., ALS) involved in 2,3-BD production and (2) stimulate the metabolism of bacteria (Garg and Jain 1995; Ji et al. 2009b; Jiayang et al. 2006; Ma et al. 2009; Sikdar and Kim 2010). For example, using an experimental design approach, Jiayang et al. (2006) tested the influence of $(NH_4)_2HPO_4$ on the yield of 2,3-BD. Increasing the $(NH_4)_2HPO_4$ concentration 4 times (up to 24 g/L) in the presence of *K. pneumoniae* CICC 10011 (5% (v/v) of inoculum), the 2,3-BD yield enhanced (from 0.02 to 0.39 g 2,3-BD/g glucose). The fermentation conditions were: 37ºC and pH 6.0 in a 0.1 L of fermentation medium supplemented with 135 g/L of glucose during 55 h.

On the other hand, using an experimental design approach with response surface analysis, Gao et al. (2010) studied the influence of K_2HPO_4 and inulin concentrations on the 2,3-BD yield in the presence of *P. polymyxa* ZJ-9 (8% (v/v) of inoculum) in a working volume of 3 L. A variation of the K_2HPO_4 and inulin concentrations (from 1 to 3.1 g/L and from 60 to 77 g/L respectively), improved the 2,3-BD yield by around 30% (from 0.37 to 0.48 g 2,3-BD/g inulin) at 30ºC and pH 6.0; the agitation rate was 240 rpm from 0 to 24 h and 120 rpm from 24 to 48 h (Gao et al. 2010).

c) Metal ions

As phosphate, metal ions affect the activity of enzymes like ALS, ALDH and BDH and stimulate the metabolism of bacteria enhancing the 2,3-BD yield (Garg and Jain 1995; Ji et al. 2009b; Jiayang et al. 2006; Ma et al. 2009; Sikdar and Kim 2010). For example, Wong et al. (2012) tested the metal ions concentration (Mn^{2+} and Co^{2+}) influence on the 2,3-BD productivity in the presence of *Klebsiella* sp. Zmd30 (2% (v/v) of inoculum) at 30ºC and 200 rpm in a 0.1 L of fermentation medium supplemented with 160 g/L of glucose (the 2,3-BD yield was not mentioned). The 2,3-BD productivity was almost tripled (from 0.41 to 1.15 g 2,3-BD/(L•h)) when the concentration of $MnCl_2.4H_2O$ was decreased from 2.2 to 0.7 mg/L

and the $CoCl_2.6H_2O$ concentration was increased from 5.3 to 5.7 mg/L. The effect of Mg^{2+} on the 2,3-BD yield was studied using a surface response experiment design in the presence of *K. pneumoniae* CICC 10011 (5% (v/v) of inoculum) at 37ºC and pH 6.0 in a 0.1 L of fermentation medium supplemented with 135 g/L of glucose for 55 h. A $MgSO_4.7H_2O$ concentration of around 0.9 g/L provided a 2,3-BD yield of 0.39 g 2,3-BD/g glucose; without Mg^{2+}, the 2,3-BD yield was 0.16 g 2,3-BD/g glucose (Jiayang et al. 2006).

Nevertheless, not all the strains are affected by metal ions. For example, Gao et al. (2010) reported that the concentration of Mn^{2+} had no effect on 2,3-BD yield. A similar conclusion was observed by Ji et al. (2009b). Therefore, metal ions could increase the 2,3-BD yield, but their influence seems to depend on the strain.

d) Acetic acid

Acetic acid increases the concentration of protons which participate in the transformation of acetoin (2,3-BD precursor) to 2,3-BD (Li et al. 2013; Nakashimada et al. 2000). For example, Wang et al. (2016) studied the influence of acetic acid on 2,3-BD yield. The fermentation of xylose (90 g/L) was performed with an acetic acid concentration varying between 0 and 6.5 g/L in the presence of *K. pneumoniae* HR521 LDH (2% (v/v) of inoculum) at 37ºC, pH 6.5 and 200 rpm in a 0.1 L of culture medium for 48 h of fermentation. In the range from 0 to 4.5 g/L of acetic acid, the 2,3-BD yield increased by 22% (from 0.32 to 0.39 g 2,3-BD/g xylose). From 4.5 to 6.5 g/L of acetic acid, the 2,3-BD yield was constant. In addition, a mixture of acetic acid (2.5 g/L) and YE (35.2 g/L) in the presence of *K. pneumoniae* HR521 LDH (5% (v/v) of inoculum) for 36 h at 37ºC, pH 6.5, 300 rpm and 2.5 vvm in a working volume of 4 L (90 g/L of xylose), raised the 2,3-BD yield up to 0.47 g 2,3-BD/g xylose (Wang et al. 2016).

The same conclusion was observed by Anvari and Motlagh (2011) using an experimental design in the presence of *K. oxytoca* PTCC 1402 (8 g/L of inoculum) at 37ºC, pH 6.0 and 150 rpm in a 0.1 L of fermentation medium containing 6 g/L of glucose. The use of acetic acid at a concentration of 0.5 g/L enhanced the 2,3-BD yield up to 0.47 g 2,3-BD/g glucose (Anvari and Motlagh 2011). However, the acetic acid can be inhibitory for bacteria like E. coli (García et al. 2013; Wlaschin and Hu 2006).

2.6.1.4 Influence of the temperature

The temperature of the fermentation medium affects the enzymatic activity and therefore the growth of microorganisms (Yang et al. 2011). Metabolic activity of microorganisms decreases at a low temperature increasing the fermentation time. On the other hand, an enzymatic denaturation or a cellular breakdown can occur at a high temperature (e.g., > 50ºC in the presence of mesophilic microorganisms) thus preventing the 2,3-BD synthesis (Garg and Jain 1995).

In order to produce 2,3-BD, most of studies tested mesophilic microorganisms able to grow in a temperature range between 20 and 50ºC; the optimal temperature range was usually between

30 and 40ºC (Boundless 2016a; Xiao et al. 2012). For example, *E. coli*, a mesophilic microorganism, grows between 21 and 49ºC, having its optimal growth temperature in the vicinities of 37ºC (Ferrer et al. 2003).

The growth temperature may be similar under different conditions for a bacterial genus like *Bacillus*. For example, 150 g/L of glucose (0.05 L of culture medium) was fermented between 25 and 40ºC in the presence of *B. amyloliquefaciens* B10-127 (4% (v/v) of inoculum) at an initial pH of 6.5 and 160 rpm. At 25ºC, the 2,3-BD yield was 0.18 g 2,3-BD/g glucose after 172 h of fermentation. However, a maximum 2,3-BD yield of 0.39 g 2,3-BD/g glucose (117% higher than at 25ºC) was obtained at 37ºC after 82 h of fermentation (Yang et al. 2011). A similar temperature (37ºC) was reported by Perego et al. (2003). A maximum 2,3-BD yield of 0.11 g 2,3-BD/g glucose (calculated from data provided by the authors) was obtained in the presence of *B. licheniformis* NCIMB 8059 (0.5 g/L of inoculum) in a working volume of 1.5 L containing 20 g/L of glucose at a pH of 6.0 and 150 rpm (undefined time). On the other hand, a temperature superior to 40ºC could also be appropriate for the bacterial growth of some mesophilic bacteria. *B. subtilis* 168 (2% (v/v) of inoculum), was grown from 37 to 50ºC at an initial pH of 6.5, 100 rpm and 10 g/L of glucose in 0.1 L (time undefined). Between 37 and 46ºC, a 2,3-BD yield of 0.35 g 2,3-BD/g glucose was obtained (calculated from data provided by the authors); the yield decreased to 0.25 g 2,3-BD/g glucose at 50°C (Fu et al., 2016).

2.6.1.5 Influence of the pH

During fermentation, the production of alcohols like 2,3-BD increases under acid conditions, whereas the production of organic acids is induced under alkaline conditions (Garg and Jain 1995). In this way, if the pH of the fermentation medium is not controlled, the carbon source is transformed into organic acids like acetic acid and succinic acid and, consequently, the medium becomes acid (Lu et al. 2012; Van Houdt et al. 2007; Xu et al. 2014). In order to prevent the intracellular bacterial acidification, the formation of alcohols like 2,3-BD increases (Hazeena et al. 2016). Hence, the pH of the fermentation medium activates or deactivates the enzymes involved in the different metabolic pathways (e.g. ALS, ALDH and BDH enzymes involved in the 2,3-BD formation) (Celińska and Grajek 2009; Garg and Jain 1995; Hazeena et al. 2016; Priya et al. 2016; Voloch et al. 1985; Xu et al. 2014; Yang et al. 2011).

A pH close to 7.0 is usually required by bacteria like *Enterobacter* or *Escherichia* to generate a maximum 2,3-BD yield. For example, *E. cloacae* SG1 (2% (v/v) of inoculum) grew in a starting pH ranged between 5.0 and 8.0 at 30ºC, 200 rpm and 30 g/L of glucose for 48 h. In the range of pH from 5.0 to 6.5, the 2,3-BD yield improved from 0.02 to 0.47g 2,3-BD/g glucose. However, in the range of pH from 6.5 to 8.0, the 2,3-BD yield declined by 53% (from 0.47 to 0.22 g 2,3-BD/g glucose) (Hazeena et al. 2016). A pH close to 7.0 was also observed for *E. coli* YJ3 (10% (v/v) of inoculum). A maximum 2,3-BD yield of 0.38 g 2,3-BD/g glucose was obtained at a pH of 6.5, 37ºC, 1.5 vvm and 400 rpm with 80 g/L of glucose for 18 h (Tong et al. 2016).

However, some authors obtained the maximum yield of 2,3-BD under acid or alkaline pH. A maximum 2,3-BD yield of 0.40 g 2,3-BD/g arabinose was obtained at a pH of 5.1, 30ºC and 200 rpm for 32 h with *E. cloacae* NRRL B-23289 (4% (v/v) of inoculum) in the presence of 50 g/L of arabinose (Saha and Bothast 1999). On the other hand, the maximum 2,3-BD yield

(0.43 g 2,3-BD/g glucose) was obtained at a pH of 8.0 for 18 h in the presence of *E. coli* BL21 (10% (v/v) of inoculum) at 1 vvm and 400 rpm (glucose concentration undefined) (Xu et al. 2014).

2.6.1.6 Influence of the fermentation time

The diol 2,3-BD, a secondary metabolite produced by bacteria and not involved in the bacterial development, is generated during the stationary phase, i.e., when the substrate begins to be depleted (Boundless 2016b; Köpke et al. 2011; UNAC 2017).

The fermentation time depends on the beginning of the stationary phase inherent to each bacteria. In addition, the fermentation time depends also on: (1) the concentration of the carbon source in the culture medium, (2) the pH, (3) the temperature, etc. For example, the growths of *K. oxytoca* ME-UD-3 and *K. oxytoca* ME-XJ-8 were compared in terms of 2,3-BD yield at 37ºC, pH 6.5, 1 vvm and 200 rpm and 5% (v/v) of inoculum for 60 h in a culture medium of 2 L containing 200 g/L of glucose. *K. oxytoca* ME-XJ-8 grew faster and achieved the stationary phase 8 h in advance (40 h) compared to *K. oxytoca* ME-UD-3 (48 h). But the yields of 2,3-BD were quite similar (around 0.45 g 2,3-BD/g glucose) (calculated from data provided by the authors) (Ji et al. 2010).

The stationary phase could be delayed increasing the fermentation time and even reducing the yield of 2,3-BD at a high substrate concentration compared to its threshold. For example, Ui et al. (1997) studied the influence of different concentrations of glucose between 5 and 100 g/L on the maximum 2,3-BD yield in the presence of *E. coli* JM109 (2% (v/v) of inoculum) at 30ºC, pH 7.5 and 160 spm. The 2,3-BD yield increased from 0.20 to 0.27 g 2,3-BD/g glucose between 5 and 40 g/L of glucose (fermentation times of 17 and 24 h respectively). Nevertheless, for a glucose concentration ranging from 70 to 100 g/L, the 2,3-BD yield slightly dropped to 0.20 and 0.17 g 2,3-BD/g glucose respectively, for fermentation times varying between 48 and 58 h. The maximum 2,3-BD yield at 40 g/L of glucose was 37% higher than the maximum 2,3-BD yield using 100 g/L of glucose; whereas the fermentation time increased by 2.4 fold in the presence of 100 g/L of glucose (Ui et al. 1997). Fermenting a high glucose concentration not only could decrease the 2,3-BD yield but also increase the fermentation time.

Parameters like pH and temperature could also influence the fermentation time. For example, Petrov and Petrova (2009) tested the pH influence on the fermentation time. In the presence of *K. pneumoniae* G31 (10% (v/v) of inoculum) at an initial pH of 8.0, 37ºC, 0.44 vvm, 200 rpm in a fed-batch fermentor (volume undefined) using glycerol as substrate, a 0.36 g 2,3BD/g glycerol yield was obtained after 280 h of fermentation. In comparison, the fermentation time decreased by 16% (280 to 234 h) at an initial pH of 5.2, for a 2,3-BD yield of 0.38 g 2,3-BD/g glycerol.

2.6.1.7 Influence of agitation

Facultative anaerobic microorganisms, such as *E. coli*, can take energy either by aerobic respiration or by fermentation in low aeration conditions (Sinha et al. 2015; Yang et al. 2011).

A low agitation rate leads to a fermentative process since agitation is linked to the dissolved O_2 (DO) concentration in the culture medium (Xu et al. 2014; Yang et al. 2011). The diol 2,3-BD is a fermentative product; a high agitation rate would decrease the production of 2,3-BD because higher amount of O_2 would be transferred to the culture medium causing the bacterial respiration (Voloch et al. 1985; Yang et al. 2011). Although, a low agitation decreases DO in the culture medium and enhances the fermentative process, moderate agitation generally improve the formation of 2,3-BD.

An agitation rate between 200 and 500 rpm in 1 L of culture medium was studied in the presence of *Klebsiella oxytoca* KMS005 (0.033 g/L of inoculum) for 48 h at 37ºC, pH 6.0, 1 vvm and 100 g/L of maltodextrin. At 200 rpm, the 2,3-BD yield was 0.32 g 2,3-BD/g maltodextrin; whereas at 300 rpm, the 2,3-BD yield increased by 16% (0.37 g 2,3-BD/g maltodextrin). Nevertheless, at 500 rpm, the 2,3-BD yield decreased by 35% compared to 300 rpm (Chan et al. 2016). On the other hand, the influence of agitation rate from 100 to 400 rpm in 0.05 L of culture medium containing 50 g/L of arabinose was studied in the presence of *Enterobacter cloacae* NRRL B-23289 (4% (v/v) of inoculum) at 30ºC and pH 5.0. The highest 2,3-BD yield was of 0.39 g 2,3-BD/g arabinose at 100 rpm and 144 h of cultivation; whereas at 300 rpm, the 2,3-BD yield decreased by 8% (0.36 g 2,3-BD/g arabinose) after 24 h of cultivation (Saha and Bothast 1999).

In the presence of *E. coli* BL21 (10% (v/v) of inoculum) in 0.8 L of culture medium (glucose concentration undefined); a 2,3-BD yield of 0.44 g 2,3-BD/g glucose was obtained. The 2,3-BD yield was 19% higher at 400 rpm than at 200 rpm for a cultivation of 17 h at a pH of 7.0 and 1 vvm (Xu et al. 2014). Using an experimental design approach, Tong et al (2016) found that the fermentation of 80 g/L of glucose in the presence of *E. coli* YJ3 (10% (v/v) of inoculum) for 18 h (2 L of culture medium) gave a 2,3-BD yield of 0.38 g 2,3-BD/g glucose at 400 rpm, 200% higher than at 200 rpm (aeration at 2 vvm). The operating conditions were 37ºC, pH 6.5 and 1.5 vvm.

Some authors varied the agitation rate in order to increase the formation of 2,3-BD. This variation consists in 2 steps: (1) a high agitation rate at the beginning of the culture favoring the bacterial growth and (2) a reduction of the agitation forcing the microaerobic conditions (a decrease of DO occurs) to improve the formation of 2,3-BD (Ji et al. 2009a; Priya et al. 2016). For example, varying the agitation rate from 300 rpm (0-15 h) to 200 rpm (15-68 h) in the presence of *Klebsiella oxytoca* ME-UD-3 (5% (v/v) of inoculum) at 37ºC, pH 6.5, 1 vvm during the cultivation of 200 g/L of glucose in 2 L of culture medium increased slightly the 2,3-BD yield by 6% (0.48 g 2,3-BD/g glucose for 56 h of cultivation) in comparison to a constant agitation of 200 rpm for 64 h (Ji et al. 2009a). The 2,3-BD yield enhancement was also observed by varying the agitation (200 rpm up to 10 h and 150 rpm from 10 to 80 h) in the presence of *Enterobacter cloacae* TERI BD18 (2% (v/v) of inoculum) at 37ºC and pH 7.5 in 100 L of culture medium (substrate concentration undefined). Priya et al. (2016) found a 2,3-BD yield 17% higher (0.48 g 2,3-BD/g glucose for 50 h of cultivation) compared to a constant agitation rate of 200 rpm for 60 h (Priya et al. 2016).

Agitation provides DO in the culture medium leading to microaerobic or aerobic conditions. It seems to be that keeping a moderate agitation (200-400 rpm for bioreactor volumes varying between 1 L and 150 L) is appropriate to maximize the 2,3-BD yield. However, a variation of the agitation rate could improve the 2,3-BD yield.

2.6.1.8 Influence of the aeration

The enzymes involved in the formation of 2,3-BD depend on the oxygen (O_2) because it inactivates ALS and BDH enzymes, reducing the 2,3-BD yield (Celińska and Grajek 2009; Chan et al. 2016; Voloch et al. 1985; Xu et al. 2014). The aeration injects air in the culture medium and thus the DO increases (Betts et al. 2014; Somerville and Proctor 2013). If the DO in the culture medium is high, the bacterial respiration is favored (Alexeeva 2000; Chan et al. 2016; Yang et al. 2011). Under anaerobic conditions, the fermentation favors the production of 2,3-BD. However, under microaerobic conditions respiration and fermentation are activated in the presence of facultative anaerobic microorganisms like *E. coli* (Alexeeva et al. 2002; Converti et al. 2003; Hanly and Henson 2013; Nakashimada et al. 2000; Xu et al. 2014). The microaerobic conditions activate the enzymes involved in 2,3-BD production because those conditions limit the bacterial respiration (Chan et al. 2016; Voloch et al. 1985).

Different aeration rates were studied in the presence of *K. oxytoca* KMS005 (0.033 g/L of inoculum) at 37ºC, pH 6.0 and 200 rpm fermenting 100 g/L of maltodextrin in 1 L of culture medium for 48 h (Chan et al. 2016). Under anaerobic conditions, a 2,3-BD yield of 0.03 g 2,3-BD/g maltodextrin was reached, which doubled under an aeration rate of 0.1 vvm. The maximum 2,3-BD yield (0.35 g 2,3-BD/g maltodextrin) was obtained at 1.2 vvm (Chan et al. 2016).

A maximum 2,3-BD yield can also be reached at a low aeration rate (close to full anaerobic conditions). An aeration range between 1.0 and 0.02 vvm was tested in the presence of *B. subtilis* 168 (2% (v/v) of inoculum) in 2.3 L of working volume containing 105 g/L of glucose at 37ºC, initial pH of 6.5 and 300 rpm (Fu et al. 2016). From 1 to 0.02 vvm, the yield of 2,3-BD and the fermentation time tripled (0.16 to 0.49 g) and doubled (40 to 86 h), respectively. The fermentation time increased because *B. subtilis* being an aerobic organism its metabolism is slower under anaerobic conditions (Fu et al. 2016).

It is also possible to perform a variation of the aeration rate during the fermentation in order to improve the 2,3-BD yield. This variation consists in 2 steps: (1) using an aeration rate to increase the bacterial population; and (2) decreasing the aeration rate forcing the microaerobic conditions in order to increase the formation of 2,3-BD (Chan et al. 2016; Fu et al. 2016; Shi et al. 2014).

2.7 Conclusion

During the last decade, the production and consumption of dairy products increased and the weight of the dairy industry in the worldwide economy is becoming more relevant. If the production of dairy products increases, the different environmental impacts of the dairy industry are higher. The main effluent of this industry, the whey, has to be treated in order to decrease its environmental impact. In this regard, whey can be treated to reduce its BOD content before being released in the environment, for example by coagulation and flocculation. But the whey can also be valorized, on one hand extracting its main components (e.g., lactose or proteins) by a separation process like membranes and, on the other hand, transformed the lactose into value-added products (e.g., biogas or chemical products like 2,3-BD) using different types of bacteria.

Many types of bacteria (e.g., *Klebsiella pneumoniae* or *Serratia marcencens*) have been used to ferment saccharides, mainly glucose, to produce 2,3-BD. In this regard, lactose (a disaccharide) contained in the whey is a potential source of carbon, which can be transformed into glucose and galactose (monosaccharides) by means of hydrolysis and, therefore, can be used to produce 2,3-BD. This process depends on the kind of bacteria. Recently, researchers have been working with 2,3-BD wild strain producers and 2,3-BD genetically modified strains producers (e.g., *E. cloacae* TERI BD18, *K. oxytoca* ME-XJ-8, etc.) in order to obtain a high yield of 2,3-BD, i.e., doing a more efficient fermentation. Even microorganisms which do not possess the 2,3-BD pathway have been used, such as E. coli, because it is easy to perform genetic modifications in this bacteria, which could improve the transformation of saccharides into 2,3-BD.

During a fermentation, parameters like substrate concentration, macro and micro nutrients, temperature, pH, residence time, agitation and aeration influenced the 2,3-BD formation. Therefore, the success of the fermentation process is related to the optimization of these parameters.

Thus, many authors have reported a high 2,3-BD yield fermenting saccharides like glucose or sucrose in the presence of bacteria like *P. polymyxa*. However, the main obstacles for the use of a biological process have been the safety level of bacteria like *E. cloacae* or *K. pneumoniae*. The use of genetically modified strains may be an avenue to overcome this problem and promote the valorization of whey into 2,3-BD.

2.8 Acknowledgement

Michèle Heitz is grateful to le Fond Québécois de la Recherche sur la Nature et les Technologies (FRQNT) in partnership with MAPAQ-NOVALAIT (Innovation en Production et en Transformation Laitières) for the grant attributed to the research program. The authors would like to thank Professor Ryszard Brzezinski of the Department of Biology, Université de Sherbrooke for his helpful discussion and advices.

Chapter 3. Biovalorization of glucose in four culture media and effect of the nitrogen source on fermentative alcohols production by *Escherichia coli*

Avant-propos:

L'article "Biovalorization of glucose in four culture media and effect of the nitrogen source on fermentative alcohols production by *Escherichia coli*" a été publié dans le Journal "*Environmental Technology*". doi : https://doi.org/10.1080/09593330.2018.1494751. La version de l'article présenté dans le document diffère de ce qui a été publié.

TITRE: Biovalorisation du glucose dans quatre milieux de culture et effet de la source d'azote sur la production d'alcools par fermentation en présence d'*Escherichia coli*.

Title: Biovalorization of glucose in four culture media and effect of the nitrogen source on fermentative alcohols production by *Escherichia coli*.

David Fernández-Gutiérrez [a,c], Marc Veillette [a], Antonio Avalos Ramirez [b], Anne Giroir-Fendler [c], Nathalie Faucheux [a] and Michèle Heitz [a, d]

[a] Université de Sherbrooke, Department of Chemical Engineering and Biotechnological Engineering. Faculty of engineering, 2500 boul. de l'Université, Sherbrooke, (Québec), Canada, J1K 2R1

[b] Centre National en Électrochimie et en Technologies Environnementales, Shawinigan, (Québec), Canada, G9N 6V8

[c] Université Lyon 1, CNRS, UMR 5256, Institut de Recherche sur la Catalyse et l'Environnement de Lyon (IRCELYON), 2 avenue Albert Einstein, 69626 Villeurbanne, France

[d] Corresponding author: E-mail address: Michele.Heitz@USherbrooke.ca

Contribution to the document: This paper is relevant to the project since: i) the bacterial population was estimated by the colony-forming unit method for a genetically modified strain of *Escherichia coli* (*E. coli* JFR1) and compared with the optical density method, ii) the importance of the culture medium related to its composition in order to produce ABD (acetoin (A) and 2,3-butanediol (2,3-BD)) was shown in the presence of 3 glucose concentrations, and iii) the influence of nitrogen source, type and concentration, on ABD yield was studied.

Biovalorization of glucose in four culture media and effect of the nitrogen source on fermentative alcohols production by *Escherichia coli*

3.1 Résumé

Le glucose est l'un des monosaccharides les plus abondants. Il est la source de carbone la plus facile à 'consommer' par des bactéries. Dans cette étude, quatre milieux de culture (LB, M9, M63 et MOPS) ont été sélectionnés ainsi que trois solutions de glucose de concentration de 4, 12.5 et 25 g/L en présence d'une souche génétiquement modifiée d'*Escherichia coli*. Les quatre milieux de culture ont été testés pour obtenir de l'ABD (acétoïne (A) et le 2,3-butanediol (2,3-BD)). Le milieu de culture sélectionné a été le M9, le plus économique, et permettant d'atteindre des rendements en ABD de 0.22 g/g glucose, lors de la fermentation de 12.5 et 25 g/L de glucose dans le milieu de culture M9 à 37ºC, pression atmosphérique, pH initial 6.5, 100 rpm et 10% (v/v) d'inoculum. L'influence de l'azote sur le rendement en ABD a été évalué en ajoutant du nitrate de sodium ($NaNO_3$) ou de l'urée ($(NH_2)_2CO$) dans le milieu de culture M9 sous 3 concentrations d'azote (2.5, 5.0 et 7.0 g azote/L). Le rendement en ABD fut amélioré de 23% à 96 h en ajoutant de l'urée (7.0 g azote/L) dans le milieu M9 en présence de 25 g/L de glucose. Par contre, l'utilisation de $NaNO_3$ a eu un effet négligeable sur la formation d'ABD.

Mots-clefs: 2,3-Butanediol, acétoïne, milieu de culture, M9, urée.

3.2 Abstract

Glucose is one of the most abundant monosaccharides and the easiest carbon source to be consumed by heterotrophic microorganisms like bacteria. In this study, four culture media (LB, M9, M63 and MOPS) were supplemented with glucose at 4, 12.5 and 25 g/L in the presence of a genetically modified strain of *Escherichia coli*. The four culture media were tested with the purpose of obtaining ABD (acetoin (A) and 2,3-butanediol (2,3-BD)). The selected medium was M9, the cheapest culture medium, giving an ABD yield around 0.22 g/g glucose fermenting 12.5 and 25 g/L of glucose in M9 culture medium at 37ºC, atmospheric pressure, initial pH 6.5, 100 rpm and 10% (v/v) of inoculum. The influence of nitrogen on ABD yield was tested adding sodium nitrate ($NaNO_3$) or urea ($(NH_2)_2CO$) to M9 culture medium at three different nitrogen concentrations (2.5, 5.0 and 7.0 g N/L). Adding urea (7.0 g N/L) to M9 supplemented with 25 g/L of glucose improved by 23% the ABD yield at 96 h. In contrast, the use of $NaNO_3$ had no significant effect on the ABD yield.

Keywords: 2,3-butanediol, acetoin, culture medium, M9, urea.

3.3 Introduction

The agro-food wastes can cause environmental problems due to their high organic load; for instance the lignocellulosic biomass presents values of biological oxygen demand (BOD) from

131 to 1237 expressed in g O_2/kg of volatile solid at 30ºC for 28 days of incubation (Bayard et al. 2016). However, the agro-food residues, such as: i) plant fibers, for example lignocellulosic fibers from corn, sugarcane and sugar-beet; or ii) wastewaters, for example from cheese manufacturing, can be valorized since they are composed of saccharides (monosaccharides, oligosaccharides, polysaccharides) (Hazeena et al. 2016). This turns agro-food wastes to plentiful, cheap and accessible sources of carbon to be fermented and transformed into added value products like ethanol, 2,3-butanediol (2,3-BD) and 3-hydroxy-2-butanone (acetoin (A)) (Gao et al. 2013; Wu et al. 2008). In addition, biovalorization is a green technique, which respects the environmental policy and does not need high temperatures or high pressures like pyrolysis (Noumi et al. 2015).

Among the fermentative products, 2,3-BD and A (ABD) have attracted more attention because of their industrial applications (Wong et al. 2014; Xiao and Lu 2014). The diol, 2,3-BD, is widely used by chemical industries as: i) a precursor of methyl ethyl ketone (or butanone, an organic solvent) and 1,3-butadiene (for manufacturing synthetic rubber) obtained by 2,3-BD dehydration; ii) a biofuel additive due to its high heating value (27.2 kJ/g of 2,3-BD); iii) a component in perfume manufacturing; iv) an antifreeze due to its relatively low normal melting point, -60°C; and v) a raw material for the manufacturing of softening agents among other uses (Białkowska 2016; Celińska and Grajek 2009; Mazumdar et al. 2013; Wang 2013). Acetoin is widely used as: i) a flavoring agent, for instance, in dairy products due to its butter flavor; ii) a compound of perfumes due to its sweet fragrant smell; iii) a component of electronic cigarette; iv) a cleaning agent; and v) a precursor of compounds, such as 2,3,5,6-tetramethylpyrazine (Xiao and Lu 2014).

Both, 2,3-BD and A can be produced by a chemical transformation. The diol 2,3-BD can be obtained performing the hydrolysis of butene oxide at a pressure of 50 bar and a temperature varying from 160 to 220°C, with a retention time unspecified (Gräfje et al. 2000); while 2-butanone can be transformed into A using, for instance, a palladium based catalyst in a concentration up to 3.3 mmol/L in water-THF (50:50, v/v) at 25ºC and 1 atm, time unspecified (El-Qisairi and Qaseer 2002). However, the biological processes are usually selected because of environmental concerns about the reduction of fossil fuels consumption, improvements of green production technologies like fermentation, and soft operating conditions of those process (generally operating in a temperature range between 25 and 45°C, at atmospheric pressure and around neutral pH) (Xiao and Lu 2014; Xu et al. 2013). ABD can be produced by a biological transformation of saccharides like glucose by yeast (i.e. *Saccharomyces cerevisiae*) or bacteria (i.e. *Enterobacter cloacae*, *Klebsiella oxytoca* or *Serratia marcescens*) (Białkowska 2016; Xiao and Lu 2014). The mentioned bacteria naturally produce ABD. However, they possess a biosafety level 2, which means they are human pathogenic (PHAC 2017). Other bacteria (e.g., *Escherichia coli*) can be genetically modified to host the genes of metabolic pathways to produce ABD (Nielsen et al. 2010; Xiao and Lu 2014). It is interesting to create genetically modified bacteria able to produce ABD because the genetic modification of bacteria can help: i) to eliminate the metabolic pathway of other sub products like acetic acid in order to improve the production of ABD; and ii) to use bacteria like *E. coli* K12 which possess a biological safety level 1 (non-human pathogenic) but do not have the metabolic pathway to produce ABD (iGEM 2017; Mazumdar et al. 2013). In the metabolic pathways involved in ABD synthesis, glucose is first transformed into pyruvic acid (PA) (glycolysis). Then, the transformation of PA into 2,3-BD is performed by 3 enzymes: α-acetolactate

synthase (ALS), α-acetolactate decarboxylase (ALDC) and 2,3-butanediol dehydrogenase (BDH) (Xiao and Lu 2014). The enzyme ALS catalyzes the formation of α-acetolactate from PA. Following this step, ALDC transforms α-acetolactate into A, a 2,3-BD precursor (Xu et al. 2015). Finally, the enzyme BDH converts A into 2,3-BD (Xu et al. 2014).

The biological production of ABD by bacteria or yeasts is performed using a culture medium, which is composed by at least a carbon source and nutrients like nitrogen, phosphorus, sulfur, etc. (Tian et al. 2014). These nutrients might positively affect the ABD production depending on the bacteria used. For example, nitrogen sources like urea, yeast extract, tryptone or sodium nitrate, affect differently the production of ABD which may increase or decrease (Wong et al. 2012). On the other hand, the rise of nitrogen concentration might improve or hamper the ABD production fermenting diverse saccharides like sucrose, glucose or a mixture glucose:xylose (2:1, w/w) as reported in the presence of bacteria like *Klebsiella sp.* or *Paenibacillus polymyxa* (Häβler et al. 2012; Ji et al. 2009b; Wong et al. 2012).

The aim of this study was therefore to ferment glucose into ABD using a genetically modified strain of *E. coli*. Firstly, the bacterial population by the colony-forming unit (CFU) and the optical density (OD) methods of the genetically modified strain was estimated in a rich culture medium, lysogeny broth (LB) (Berney et al. 2006). Secondly, since the formation of ABD varies depending on the type and concentration of nutrients, 4 different culture media (3 minimal culture media: medium 9 (M9) (Ecocyc 2017; Maniatis et al. 1982), medium 63 (M63) (Pardee et al. 1959) and potassium morpholinopropane sulfonate medium (MOPS) (Wisconsin-University 2017) and 1 rich medium (LB) (Berney et al. 2006) were supplemented with 3 glucose concentrations (4, 12.5 and 25 g/L). Finally, in order to observe the influence of an additional nitrogen source (ANS) and its concentration on ABD formation, the effect of 2 nitrogen sources (sodium nitrate ($NaNO_3$) and urea ($(NH_2)_2CO$)) and their concentrations (with an equivalent concentration of nitrogen for both sources: 2.5, 5.0 and 7.0 g N/L) were tested.

3.4 Materials and methods

3.4.1 Microorganisms

Two strains of *E. coli* MG1655, wild (ECW) and genetically modified (ECGM), were grown in the rich culture medium LB. The ECGM strain hosts the metabolic pathway to produce ABD from *Enterobacter cloacae* (*E. coli* MG1655/ALDH+*bud*ABC, named *E. coli* JFR1). Furthermore, the production of lactate was blocked by inactivation of the enzyme lactate dehydrogenase in *E. coli* JFR1 strain. Both strains were conserved into a mixture of glycerol and lysogeny broth (LB) culture medium (50:50, v/v) at -81ºC.

3.4.2 Inoculum preparation and population estimation

The seed culture medium was prepared as follows: 0.1 mL of *E. coli* (ECW or ECGM) from the conservation blend was mixed with 9.9 mL of fresh LB culture medium into a test tube (15 mL). The test tube was incubated overnight at 37ºC to obtain the pre-culture medium. The population estimation of the inoculum was performed using 2 mL of pre-culture medium transferred to a 500 mL Erlenmeyer flask containing 200 mL of fresh LB culture medium. The

flask was incubated for 9 h in a rotary shaker incubator at 37ºC and 100 rpm. After this time (9 h), the seed culture medium obtained was used to inoculate the culture media.

Wild (ECW) and genetically modified (*E. coli* JFR1) strains were grown in LB culture medium in order to estimate and compare their bacterial population, using 9 replicates, to determine the CFU/mL of broth. In order to determine the CFU, 6 consecutive dilutions were carried out mixing 0.9 mL of fresh LB culture medium with 0.1 mL from the previous dilution to the following one. For example, in order to obtain the dilution 10^{-3}, 0.1 mL from the dilution 10^{-2} were taken and mixed with 0.9 mL of fresh LB culture medium. Then, samples of 0.1 mL were taken from the last dilution (10^{-6}), spread on LB agar plates and incubated for 9 h at 37ºC. In this way, colonies appeared on the agar surface and were directly counted.

3.4.3 Culture media and conditions

The fermentation of glucose using *E. coli* JFR1 was tested using 4 culture media (LB, M9, M63 and MOPS) and the ABD yields were compared. The composition of each culture medium is described in Table 3.1. The culture media were sterilized by autoclaving them at 121°C and 1.2 atm for 0.25 h. All experiments were performed in triplicate using Erlenmeyer flasks with a working volume of 200 mL (10% (v/v) of inoculum) at 37ºC, 100 rpm, initial pH 6.5 and atmospheric pressure unless otherwise indicated. Flasks were placed on a rotary shaker incubator (Fermentation Design inc, Allentown, PA) for 96 h. Beyond this time, the ABD yield decreased. After selecting a culture medium (M9), 2 nitrogen sources ($NaNO_3$ and $(NH_2)_2CO$) were tested for 72 h as a comparison purpose. The additional nitrogen concentration in M9 supplemented with glucose (12.5 and 25 g/L) was the same for both sources: 2.5, 5.0 and 7.0 g N/L.

Table 3.1: Composition of the 4 culture media in 1L of distilled water - LB medium (Berney et al. 2006); M9 minimal medium (Ecocyc 2017; Maniatis et al. 1982); M63 minimal medium (Pardee et al. 1959); and MOPS (3-morpholinopropane-1-sulfonic acid) minimal medium (Wisconsin-University 2017).

Culture medium	Nutrient	Concentration (g/L)
LB	Tryptone	10.00
	Yeast extract	5.00
	NaCl	10.00
M9 (supplemented with $MgSO_4 \cdot 7H_2O$ and $CaCl_2$)	$Na_2HPO_4 \cdot 7H_2O$	12.80
	KH_2PO_4	3.00
	NH_4Cl	1.00
	NaCl	0.50
	$MgSO_4 \cdot 7H_2O$	0.49
	$CaCl_2$	0.01
M63	KH_2PO_4	13.50
	$(NH_4)_2SO_4$	2.00
	$MgSO_4 \cdot 7\ H_2O$	0.20
	$FeSO_4$	5.0×10^{-4}
	MOPS	8.30

MOPS (3-morpholinopropane-1-sulfonic acid)	NaCl	2.92
	K_2HPO_4	0.23
	$MgCl_2$	0.05
	Tricine	0.72
	NH_4Cl	0.51
	K_2SO_4	0.05
	$FeSO_4 \cdot 7H_2O$	$3.0x10^{-3}$
	$CaCl_2 \cdot 2H_2O$	$1.0x10^{-4}$
	H_3BO_3	$2.5x10^{-5}$
	$MnCl_2 \cdot 4\ H_2O$	$1.6x10^{-5}$
	$CoCl_2 \cdot 6\ H_2O$	$7.0x10^{-6}$
	$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	$3.6x10^{-6}$
	$ZnSO_4 \cdot 7\ H_2O$	$2.8x10^{-6}$
	$CuSO_4$	$2.4x10^{-6}$

3.4.4 Analytical methods and reagents

The concentration of glucose and fermentative products (A, 2,3-BD, ethanol and acetic acid) were determined by high performance liquid chromatography (HPLC). Raw broth withdraw from each flask was pre-filtered in order to remove the solids using 0.2 μm pore size filters. Ten (10) μL of sample were injected into an Agilent series 1100 chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a column Aminex HPX-87H (300 x 7.8 mm) in order to separate glucose and products (2,3-BD, A, ethanol and acetic acid) at 25ºC using sulfuric acid 0.005M as a mobile phase (0.4 mL/min). Refractive index (RI) and ultraviolet (UV) detectors were used for the quantification of the products (glucose, 2,3-BD, ethanol and acetic acid concentration were quantified by RI detector; whereas acetoin concentration was estimated by UV detector).

The reagents used for the analytical method were HPLC grade. Glucose (≥ 99.5%), A and 2,3-BD (98%) were purchased from Sigma-Aldrich (Canada). Acetic acid (ACS pure) and ethanol (≥ 99%) were purchased from Fisher Scientific Inc. (Canada).

3.4.5 Statistical analysis

The significant parameters (i.e., culture medium, glucose concentration and nitrogen source and concentration) on ABD yield were determined by means of an analysis of variance (ANOVA) with a P-value lower than 0.05. Dixon's Q test was used to estimate and reject outlier values of ABD yield using a confidence level of 95% (Rorabacher 1991).

3.5 Results and discussion

3.5.1 Optical density and colony-forming units for ECW and *E. coli* JFR1 strains

The bacterial growth can be defined by 3 phases: the lag phase, the exponential phase and the stationary phase, where the maximum bacterial population is reached. Figure 3.1 presents the results of OD at 600 nm (OD_{600}) and CFU/mL as a function of the fermentation time obtained in the presence of ECW (Figure 3.1A) and *E. coli* JFR1 (Figure 3.1B) in LB culture medium. In the case of ECW, according to OD_{600}, the exponential growth phase started after 2 h (OD_{600}: 0.10) of fermentation until 4 h (OD_{600}: 0.51). For a fermentation time higher than 4 h, the OD_{600} increased slowly to reach 0.77 at 9 h. A similar tendency was observed in Figure 3.1B with the *E. coli* JFR1 strain. The exponential growth phase began after 2 h of fermentation (OD_{600}: 0.05) and increased up to 4 h (OD_{600}: 0.47). Finally, the *E. coli* JFR1 growth increased slowly to reach an OD_{600} value close to 0.82 at 9 h. Taking into account the OD_{600} values for both *E. coli* strains, both bacterial growths were similar. According to Sezonov et al. (2007), the exponential phase ends when the OD_{600} value is ranged between 0.6 and 1.0 in LB culture medium in the presence of *E. coli* K12 MG1655 at 37ºC and 180 rpm. This fact was also reported by Quigley (2008) using another *E. coli* strain (*E. coli* DH5α), where a typical OD_{600} value at 37°C in LB culture medium was 0.8 at 6 h of fermentation, the end point of the exponential growth phase. In the present study, the growth tendency for ECW and *E. coli* JFR1 could be considered similar to a typical growth curve for *E. coli* bacteria in LB culture medium with an OD_{600} close to 0.8 after 9 h of fermentation for both strains (ECW and *E. coli* JFR1).

In order to confirm the bacterial growth of each *E. coli* strain, the CFU/mL value was determined. In fact, both strains presented a relatively fast growth on LB agar plates (based on CFU/mL), where colonies appeared approximately after 9 h of incubation. In the presence of ECW (Figure 3.1A), the CFU/mL values were practically stable at $1.1x10^9$ CFU/mL for fermentation times ranging from 6 to 9 h. This fact clearly confirms that the stationary growth phase is reached after 6 h. The CFU/mL value was similar to the one obtained for *E. coli* JM109 at 37ºC and 250 rpm using LB culture medium (Matlock et al. 2017). However, the CFU/mL obtained in the present study was 39% lower ($1.8x10^9$ CFU/mL) than the value reported by Yu et al. (2006). The authors used a tryptone soy broth as a culture medium, which presents a richer composition compared to LB culture medium (Ecocyc 2017), in the presence of *E. coli* K12 for 24 h at 37°C and 140 rpm.

On the other hand, in the present study, *E. coli* JFR1 strain reached the stationary growth phase faster than ECW, since it was achieved after 5 h of fermentation. The CFU/mL value at 5 h was $6.5x10^8$ CFU/mL, 41% lower compared to the CFU of ECW probably linked to the genetic modification performed in *E. coli* JFR1, since in recombinant strains a new gene might be toxic for the bacteria and thus their growth might be lower (Rosano and Ceccarelli 2014).

The difference between OD_{600} and CFU techniques could be explained by the fact that OD_{600} is a direct technique to measure the turbidity of the broth (Sutton 2011). In the broth, there is a mixture of viable bacteria and dead bacteria. If many dead cells are in suspension, the measurement of the bacterial growth using OD_{600} might have been overestimated, whereas the

quantified bacterial population using the CFU method only takes into account the viable bacteria. Therefore, the CFU method seems to be more suitable than OD_{600} to estimate the bacterial population in the inoculum.

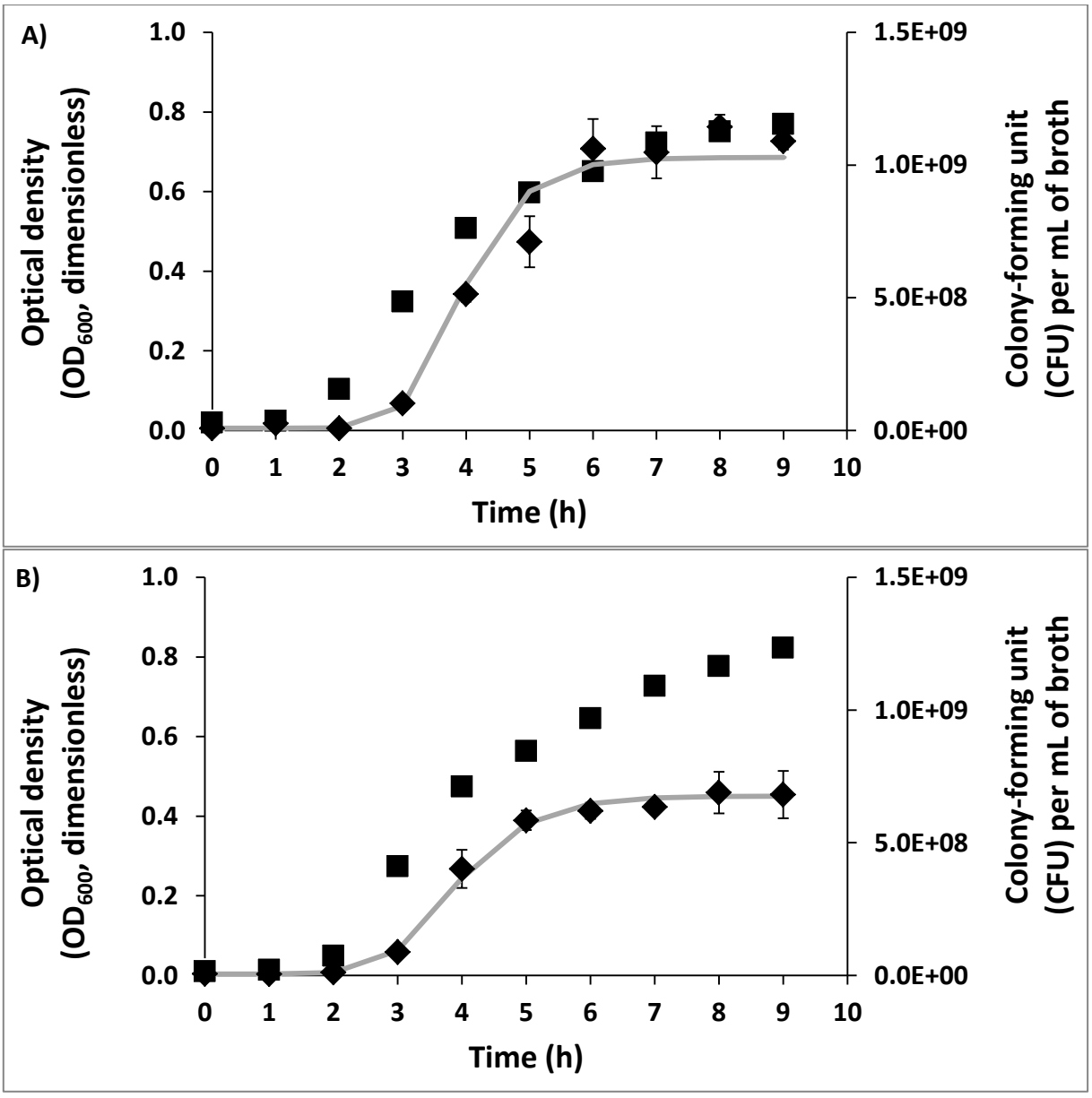


Figure 3.1: Optical density at 600nm (OD600, ■), colony-forming units per mL of broth (CFU/mL, ♦) and modeling of the growth curve by the modified Gompertz model (-) as a function of time in the presence of ECW (A) and *E. coli* JFR1 (B) strain. Results are means ± SD of 9 replications performed in flasks at 37ºC, atmospheric pressure, initial pH of 6.5 and 100 rpm a working volume of 200 mL (batch fermentation).

3.5.2 Modeling of the bacterial growth

The experimental bacterial growth data of ECW and *E. coli* JFR1 strains were modeled using the modified Gompertz model (Zwietering et al. 1990):

$$\ln\frac{N}{N_0} = K * exp\left[-exp^{(\frac{\mu_m * 2.718}{K} * (\lambda - t) + 1)}\right]$$

where N is the CFU value (CFU/mL) for a given time and N_0 the CFU value (CFU/mL) at the initial time; K is the asymptote (ln $[N_\infty/N_0]$) in the stationary phase; μ_m is the maximum specific growth rate (h^{-1}, which comes expressed as $(\ln(CFU_1) - \ln(CFU_2)) / (t_1 - t_0)$); λ is the lag time (h) and t is the time (h).

The modified Gompertz model was fitted with the experimental bacterial growth data by a non-linear regression (Figure 3.1). Determination coefficients (R^2) values of 0.981 and 0.999 were respectively obtained for the ECW and the *E. coli* JFR1 strains. The values of λ were 2.2 and 1.8 h for the ECW and *E. coli* JFR1, respectively. This fact points out that the *E. coli* JFR1 strain was able to adapt to the LB culture medium faster than ECW. However, ECW reached a higher μ_m (2.8 h^{-1}) compared to *E. coli* JFR1 (2.4 h^{-1}), achieving a higher bacterial population. Compared to the best μ_m values obtained in the presence of 2 mutants of *E. coli* MG1655 (soluble transhydrogenase deficient and Entner-Doudoroff pathway deficient) and the wild strain (37°C and 200 rpm in M9 culture medium supplemented with 5 g/L of glucose), the *E. coli* JFR1 μ_m obtained in the present study was more than 3 fold higher (Sauer et al. 2004). However, the value of *E. coli* JFR1 μ_m was similar to the one obtained by Berney et al. (2006) using LB without glucose as culture medium fermented at 37°C and 200 rpm in the presence of *E. coli* K12 MG1655 (wild strain).

3.5.3 Influence of glucose concentration supplementing 4 culture media on ABD yield in the presence of *E. coli* JFR1

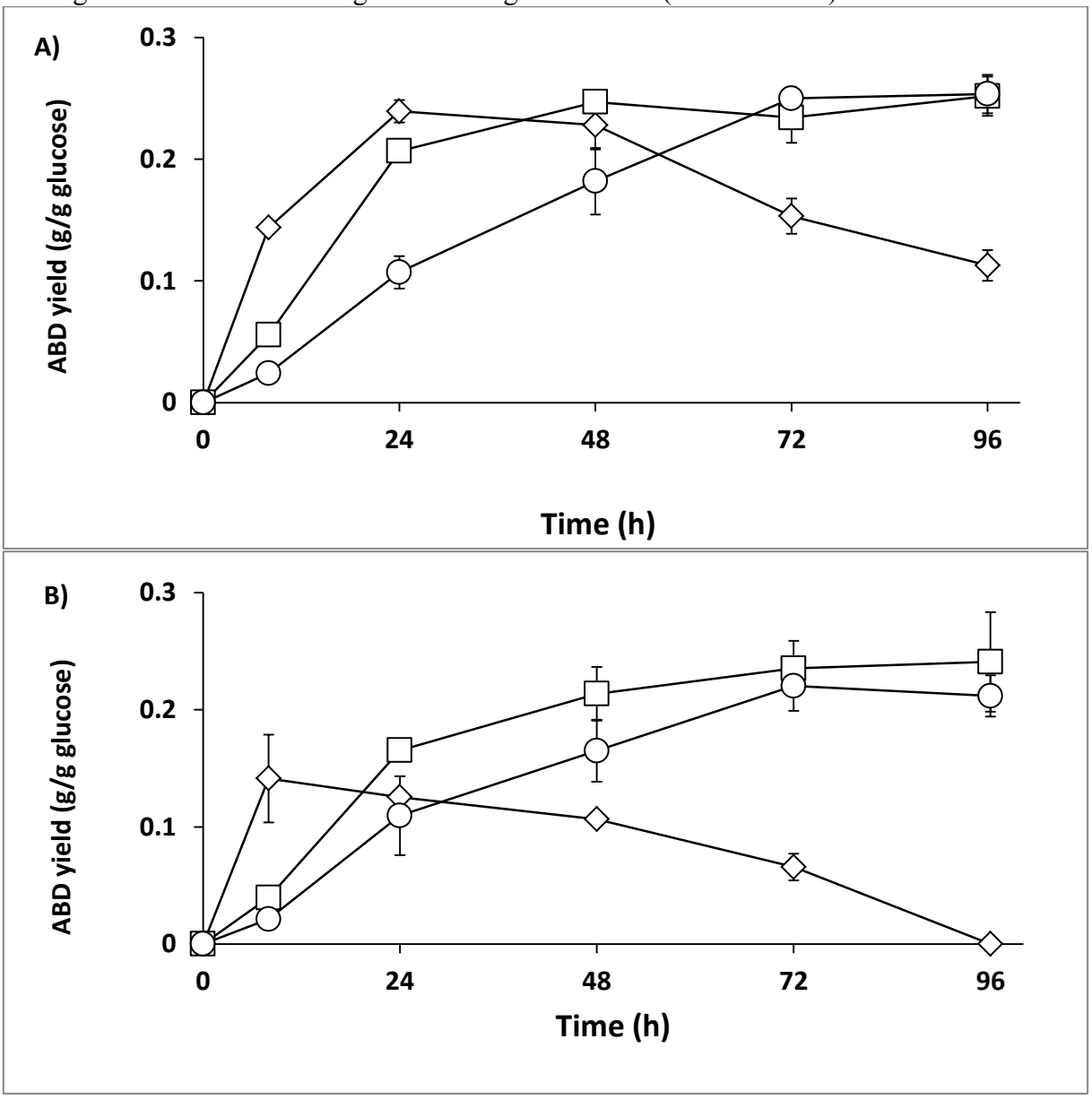
The production of ABD by *E. coli* JFR1 was studied using 4 culture media (LB, M9, M63 and MOPS) for 96 h supplemented with 4, 12.5 and 25 g/L of glucose. In most of the experimental conditions, glucose was exhausted (conversion 100%) after a maximum of 72 h. However, a maximum glucose conversion of 92% after 96 h was observed using M9 culture medium supplemented with 25 g/L of glucose. In the same way, by fermenting 12.5 and 25 g/L of glucose in MOPS culture medium, a maximum glucose conversion of 93% and 66% respectively were observed after 96 h (Table 3.2).

Table 3.2: Maximum glucose conversion (%) as a function of glucose concentration (g/L) and time (h) in the 4 studied culture media at 37ºC, atmospheric pressure, initial pH of 6.5, 100 rpm and 10% (v/v) of inoculum in a working volume of 200 mL (batch fermentation).

Culture medium	Glucose concentration (g/L)	Time (h)	Glucose conversion (%)
LB	4.0	24	100
	12.5	48	100
	25.0	72	100
M9	4.0	24	100
	12.5	48	100
	25.0	96	92
M63	4.0	8	100
	12.5	24	100
	25.0	72	100
MOPS	4.0	24	100
	12.5	96	93
	25.0	96	66

Figure 3.2A presents the ABD yield as a function of fermentation time using LB, a rich culture medium formed by tryptone, yeast extract and sodium chloride, supplemented with 3 glucose concentrations (4, 12.5 and 25 g/L). The maximum ABD yield was around 0.25 g ABD/g glucose independently of the glucose concentration. Therefore, the increase of glucose concentration had no influence on the maximum ABD yield using LB culture medium ($p < 0.05$), but the fermentation time to obtain the maximum ABD yield increased according to the glucose concentration, ranging from 24 h (4 g/L of glucose) to 72 h (25 g/L of glucose). On the other hand, after 24 h of fermentation, the ABD concentration decreased when LB culture medium was supplemented with 4 g/L of glucose (Figure 3.2A). As suggested by Mazumdar et al. (2013), once the carbon source (glucose) is depleted, bacteria could consume another carbon source like ABD during the stationary growth phase, causing a drop of its yield. This fact is observed in Figure 3.2A and thus it seems to confirm that *E. coli* JFR1 consumes ABD as a carbon source in order to maintain its metabolic activity. Lysogeny broth (LB) is a rich culture medium, which provides all nutrients (carbon, nitrogen, phosphorus, etc.) required by bacteria (Ecocyc 2017). In this way, the components present in LB culture medium like amino acids (e.g. leucine, methionine and tryptophan) could positively influence the ALS and ALDC enzymes (Cho et al. 2015). This fact may explain the similar ABD yields obtained whatever the glucose concentration, since the LB culture medium nutrients might efficiently stimulate the formation of ABD even at a relatively low glucose concentration.

Figures 3.2B and 3.2C present the ABD yield for *E. coli* JFR1 as a function of the fermentation time using M9 and M63 as culture media, respectively. When both culture media were supplemented with 4 g/L of glucose, the maximum ABD yield was obtained after 8 h of fermentation. For a higher fermentation time, the ABD yield constantly decreased down to zero (under the detection limit of the HPLC) since it was probably used as a carbon source. For both culture media, similar maximum ABD yield was obtained at 72 h using M9 (0.22 g ABD/g glucose) and M63 (0.25 g ABD/g glucose) in the presence of 12.5 and 25 g/L of glucose. The similar ABD yields obtained at 72 h for glucose concentrations of 12.5 and 25 g/L may be due to the inhibitory effects occasioned on *E. coli* JFR1 by a relatively high concentration of glucose (Tian et al. 2014), which might be close to 25 g/L for *E. coli* JFR1. The inhibitory effect of glucose concentration was also observed, for instance, in the presence of *E. coli* JM109 (2% (v/v) of inoculum) in LB culture medium at 30°C, pH 7.5 and 160 rpm when glucose concentration higher than 40 g/L was used (Ui et al. 1997).



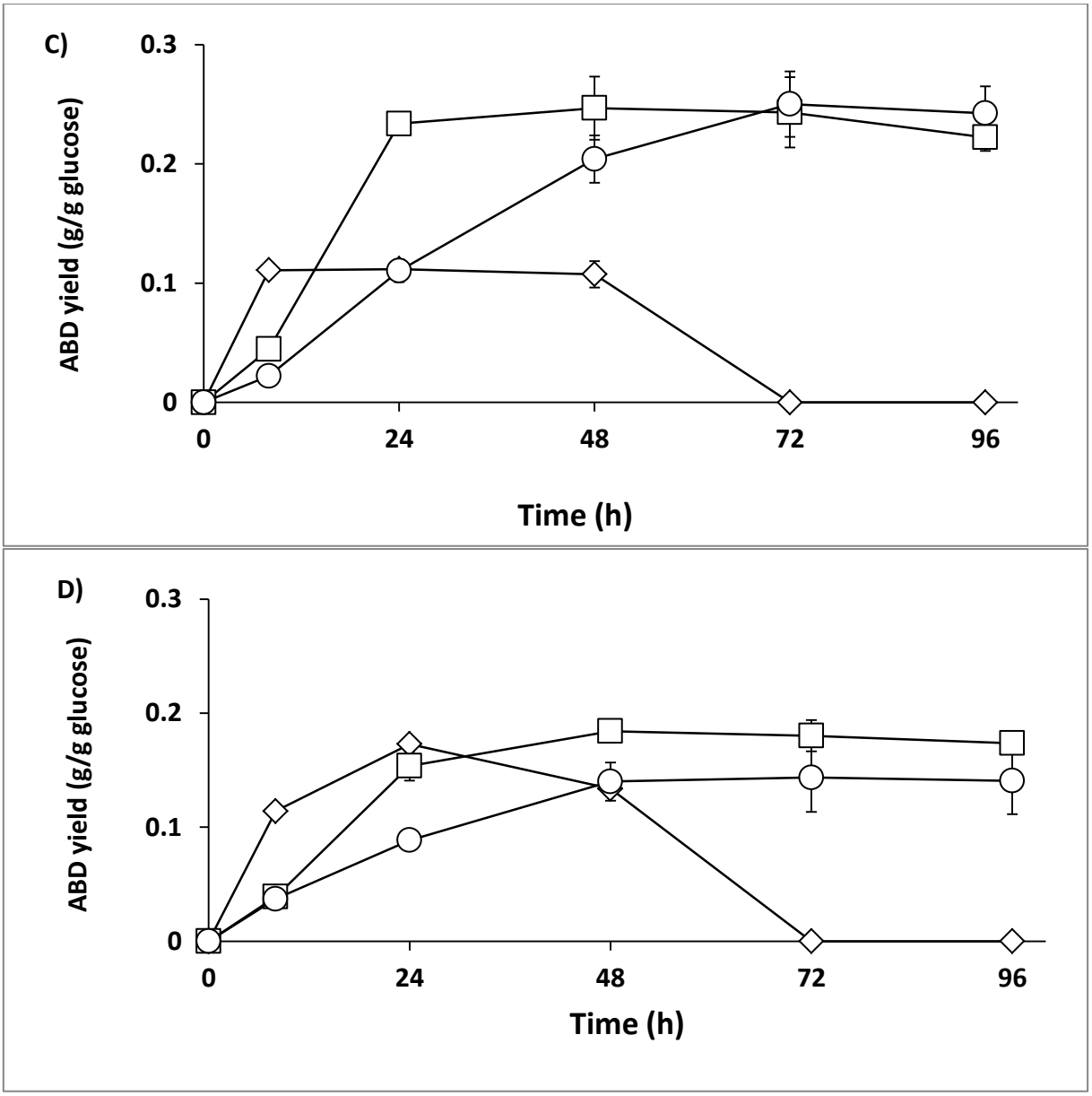


Figure 3.2: ABD yield as a function of time in the presence of *E. coli* JFR1 in LB (A), M9 (B), M63 (C) and MOPS (D) culture media at 4 g/L glucose (◊) , 12.5 g/L of glucose (□) and 25 g/L of glucose (○). Results are means ± SD of 3 replications performed in flasks at 37ºC, atmospheric pressure, initial pH of 6.5, 100 rpm and 10% v/v of inoculum in a working volume of 200 mL (batch fermentation).

The maximum ABD yield (close to 0.25 g ABD/g glucose, at 72 h fermenting 12.5 and 25 g/L of glucose) obtained using LB, M9 and M63 as culture media was similar to the one reported in the presence of different *E. coli* strains. For instance, Ui et al. (1997) and Yan et al. (2009) reported ABD yields of 0.25 g ABD/g glucose (yield calculated using the experimental data provided by the authors) in the presence of *E. coli* JM109 (2% (v/v) of inoculum and 20 g/L of

glucose) and *E. coli* JCL260 (1% (v/v) of inoculum and 40 g/L of glucose), respectively. The fermentations were performed at 30°C, pH 7.5 and 160 rpm for 20 h using *E. coli* JM109; and at 37°C (from 0 h to 3 h) and 30°C (from 3 h to 48 h), pH 7.0 and 250 rpm using *E. coli* JCL260.

Figure 3.2D presents the ABD yield variation as a function of time using MOPS as culture medium in the presence of *E. coli* JFR1. The maximum ABD yields were similar fermenting 4 and 12.5 g/L of glucose (around 0.18 g ABD/g glucose) after 24 and 48 h, respectively. However, the maximum ABD yield was 22% smaller (0.14 g ABD/g glucose at 48h) with MOPS supplemented with 25 g/L of glucose compared to the other glucose concentrations. A 25 g/L of glucose in MOPS may cause the bacterial inhibition and explain the lower ABD yield, associated with a 66% of glucose conversion (Table 3.2). The low glucose conversion and the low ABD formation was also observed by Nielsen et al. (2010) with *E. coli* YYC202 strain (17% (v/v) of inoculum, estimated by the data provided by the authors) in LB culture medium. A maximum glucose conversion of 38% was obtained at 30°C for 120 h (pH and agitation non-defined) and the ABD yield was similar to the one of the present study (0.14 g ABD/g glucose, the yield was calculated using the experimental data provided by the authors). Moreover, MOPS contains several divalent metal ions like iron (Fe^{2+}), cobalt (Co^{2+}) and manganese (Mn^{2+}), which could influence the ABD formation (Garg and Jain 1995, Wong et al. 2012). The divalent metal ions present in MOPS culture medium might inhibit the enzymes involved in the formation of ABD, as reported by several authors (Tian et al. 2014; Wong et al. 2012; Yu et al. 2015).

The selection of a culture medium was based on the ABD yield and the respective cost of the medium. The cost of each culture medium is as follows: MOPS around 12 ($/L), M63 and LB around 8 ($/L) and M9 near 6 ($/L) (SigmaAldrich 2017). MOPS culture medium was not chosen because the lowest ABD yield among the 4 culture media tested in the presence of 12.5 and 25 g/L of glucose was obtained. LB and M63 culture media were rejected because they are more expensive and presented similar ABD yields ($p < 0.05$) than M9. In the subsequent experiments, M9 culture medium was supplemented with 12.5 and 25 g/L of glucose in order to test the effect of additional nitrogen sources on ABD yield.

3.5.4 Influence of the source and concentration of an additional nitrogen source on ABD yield in the presence of *E. coli* JFR1

Nitrogen is an important macronutrient since it is involved in the bacterial growth (constitution of coenzymes and amino acids, Todar 2017) and thus may improve the formation of ABD as shown in a study performed by Wong et al. (2012). The authors reported that depending on the source and concentration of nitrogen, the ABD yield improved. In the present study, 2 additional inorganic nitrogen sources (urea and $NaNO_3$) were supplemented to the M9 culture medium in order to test the effect of the source and the concentration of nitrogen source on ABD formation at 2 glucose concentrations (12.5 and 25 g/L). Both urea and $NaNO_3$ concentration were kept at the same nitrogen (N) concentration (2.5, 5.0 and 7.0 g N/L) in order to allow a comparison. In the case of urea, additional concentrations of nitrogen (0.6 and 1.2 g N/L in the presence of 12.5 g/L of glucose; and 10 g N/L in the presence of 25 g/L of glucose) were tested.

The use of $NaNO_3$ hampered the formation of ABD and the consumption of glucose never exceeded 6 and 8 g/L at 48 h whatever nitrogen concentration used at 12.5 or 25 g/L of glucose respectively (Table 3.3) in all fermentations. This fact may point out i) that $NaNO_3$ inhibited the bacterial growth of *E. coli* JFR1 since *E. coli* may reduce nitrate (NO_3^-) into nitrite (NO_2^-), which is toxic for the cells (Tiso and Schechter 2015); and ii) may explain the low glucose conversion (around 50% and 30% fermenting 12.5 and 25 g/L of glucose respectively).

Table 3.3: Glucose conversion (%) as a function of nitrogen concentration (g N/L) in the presence of 12.5 and 25 g/L of glucose in M9 culture medium using $NaNO_3$ as an ANS at 37ºC, atmospheric pressure, initial pH of 6.5, 100 rpm and 10% (v/v) of inoculum in a working volume of 200 mL (batch fermentation).

Nitrogen concentration (g N/L)	**Glucose concentration (g/L)**	**Maximum glucose conversion (%)**
2.5	12.5	45
5.0		49
7.0		48
Nitrogen concentration (g N/L)	**Glucose concentration (g/L)**	**Maximum glucose conversion (%)**
2.5	25	30
5.0		32
7.0		33

On the other hand, *E. coli* JFR1 produced acetic acid (yield ranged from 0.06 to 0.09 g acetic acid/g glucose between 48 and 96 h, in the presence of 12.5 g/L of glucose; and was around 0.04 g acetic acid/g glucose at 48 h, fermenting 25 g/L of glucose for all nitrogen concentrations tested) in the presence of $NaNO_3$ (Table 3.4).

Acetic acid is a fermentative product formed by *E. coli* which inhibits its growth (García et al. 2013). Hence, the production of acetic acid besides the reduction of NO_3^- prevents the formation of ABD and the consumption of glucose. The null ABD formation using $NaNO_3$ as a nitrogen source was also observed by Xiao et al. (2012) using *Geobacillus* sp. XT15 (5% (v/v) of inoculum) to ferment 20 g/L of glucose at 55°C, initial pH of 8 and 170 rpm (fermentation time unspecified). However, the authors reported an ABD yield of 0.40 g ABD/g glucose under the same operating conditions using a mixture of 20 and 10 g/L of peptone and yeast extract respectively.

Table 3.4: Acetic acid yield (g/g glucose) as a function of nitrogen concentration (g N/L) in the presence of 12.5 and 25 g/L of glucose in M9 culture medium using $NaNO_3$ as an ANS at 37ºC, atmospheric pressure, initial pH of 6.5, 100 rpm and 10% v/v of inoculum in a working volume of 200 mL (batch fermentation).

Nitrogen concentration (g N/L)	**Glucose concentration (g/L)**	**Acetic acid yield (g/g glucose)**
2.5	12.5	0.06
5.0		0.09
7.0		0.08
Nitrogen concentration (g N/L)	**Glucose concentration (g/L)**	**Acetic acid yield (g/g glucose)**
2.5	25	0.03
5.0		0.04
7.0		0.04

Figures 3.3A and 3.3B present the glucose conversion and ABD yield, respectively, as a function of time in the presence of 12.5 g/L of glucose. Urea was used as an ANS. Two additional nitrogen concentrations (0.6 and 1.2 g N/L) were also tested.

Glucose was exhausted after 24 h of fermentation when urea was added to M9 culture medium at nitrogen concentrations ranged from 2.5 to 7.0 g N/L, whereas glucose was totally consumed in the presence of 0.6 and 1.2 g N/L after 48 h (Figure 3.3A). After 24 h, the ABD yield increased by 21% and 17% in the presence respectively of 2.5 and 5.0 g N/L, in comparison of the ABD yield obtained without ANS (0.17 g ABD/g glucose, Figure 3.2B). However, adding 0.6, 1.2 and 7.0 g N/L did not improve the ABD yields, (0.14, 0.16 and 0.15 g ABD/g glucose) respectively (Figure 3.3B). The highest ABD yield, 0.26 g ABD/g glucose, was reached in the presence of 1.2 g N/L after 48 h, increasing by 29% compared to the one achieved without ANS. In all cases, the ABD yield remained practically constant once the highest yield was reached for each nitrogen concentration.

The formation of acetic acid, an inhibitory compound for *E. coli*, was not detected in the presence of urea fermenting 12.5 g/L of glucose (data not shown). Figure 3.3B presents the highest and lowest ethanol yields which were obtained with 0.6 g N/L and without ANS respectively. Ethanol was produced in a range varying between 0.07 and 0.11 g ethanol/g glucose for all the nitrogen concentrations.

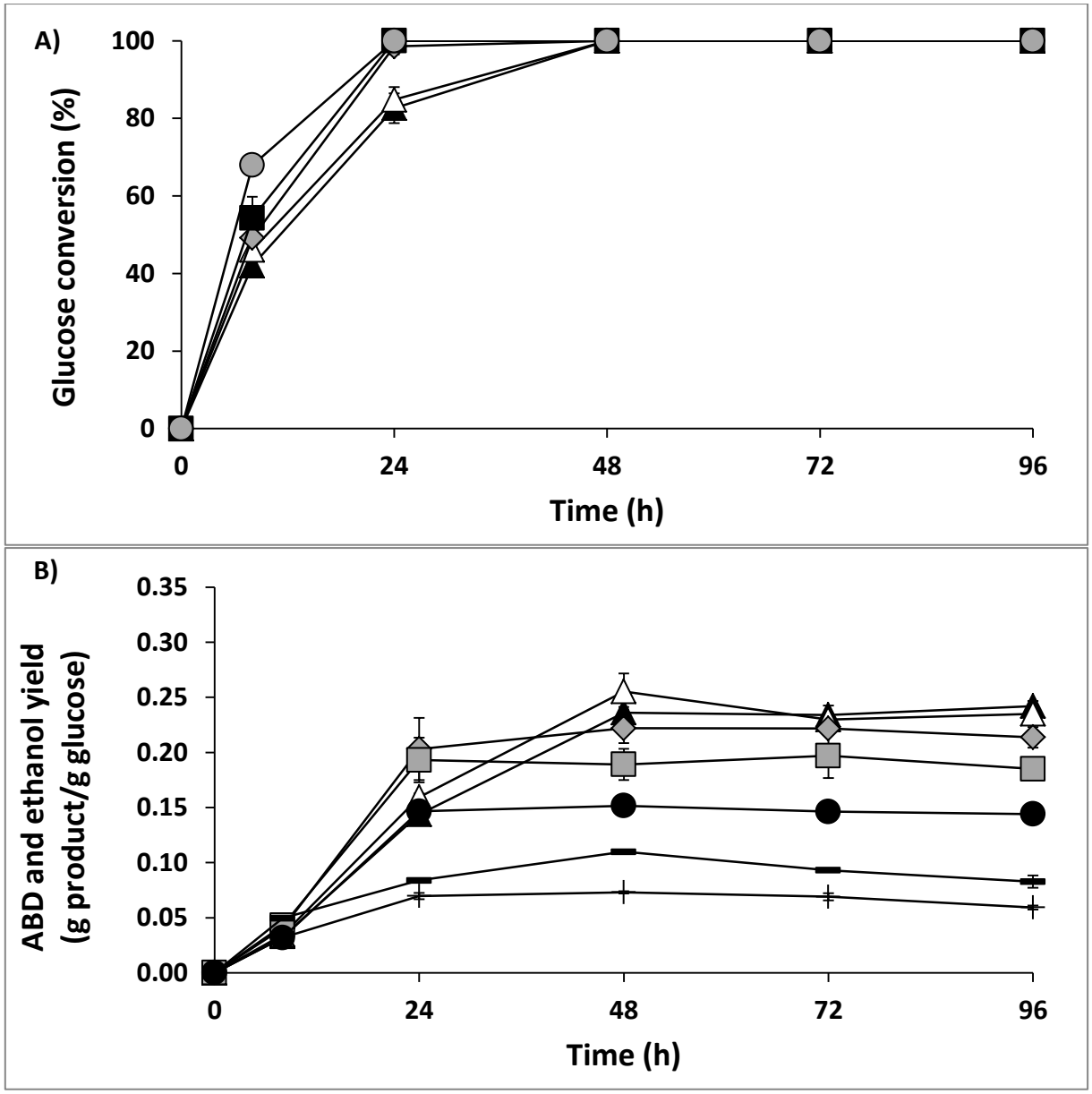
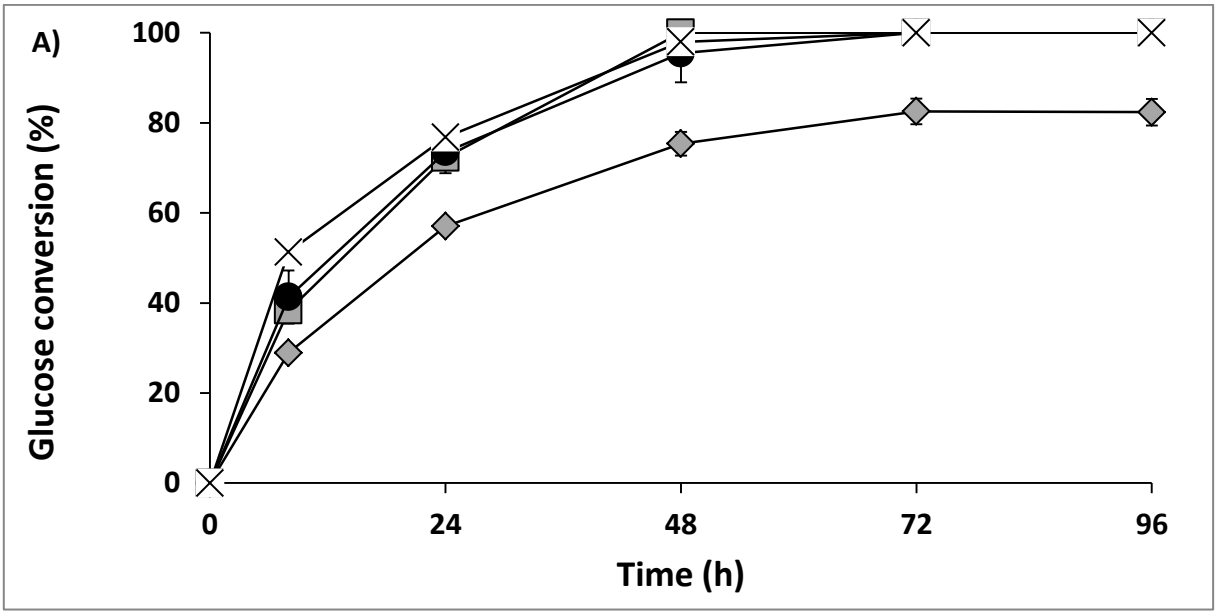


Figure 3.3: Glucose conversion (A) and ABD yield (B) in M9 culture medium supplemented with 12.5 g/L of glucose as a function of time in the presence of *E. coli* JFR1. Source of nitrogen: urea at 0.6 g N/L (▲), 1.2 g N/L (Δ), 2.5 g N/L (♦), 5.0 g N/L (■) and 7.0 g N/L (●). Additionally, the highest (▬) and lowest (+) ethanol yields obtained for 0.6 g N/L and without an ANS are represented respectively. Results are means ± SD of 3 replications performed in flasks at 37ºC, atmospheric pressure, initial pH of 6.5, 100 rpm and 10% v/v of inoculum in a working volume of 200 mL (batch fermentation).

Figures 3.4A and 3.4B show the glucose conversion and ABD yield, respectively, as a function of time when glucose (25 g/L) was fermented in the presence of *E. coli* JFR1 and urea as an ANS. The preliminary fermentations showed that increasing the nitrogen concentration provided a higher ABD yield. For this reason, a higher urea concentration (10.0 g N/L) was also tested. A 100% glucose conversion was reached at 48 h when 5.0 7.0 and 10.0

g N/L were used; whereas the glucose conversion was 75% in the presence of 2.5 g N/L. The 2.5 g N/L had no significant effect on the ABD yield (0.15 g ABD/g glucose) compared to the experiments performed without ANS (0.15 g ABD/g glucose) at a fermentation time of 48 h. However, the ABD yields compared to the one obtained in absence of urea increased by 66%, 47% and 41% using 5.0, 7.0 and 10.0 g N/L, respectively at 48 h. The highest ABD yield (0.27 g ABD/g glucose) was achieved at 7.0 g N/L after 96 h of fermentation. No acetic acid production was detected fermenting 25 g/L of glucose in the presence of 7.0 and 10.0 g N/L; whereas in the presence of lower nitrogen concentration like 2.5 and 5.0 g N/L acetic acid was produced (up to 0.06 and 0.02 g acetic acid/g glucose) at 96 h (data not shown). Therefore, the formation of acetic acid was avoided increasing the urea concentration (7.0 and 10.0 g N/L) in the presence of 25 g/L of glucose. Figure 3.4B presents the highest and lowest ethanol yields obtained for 10.0 and 2.5 g N/L respectively, where the ethanol yield was nearly constant, ranging from 0.04 to 0.07 g ethanol/g glucose when a concentration of 25 g/L of glucose was fermented.



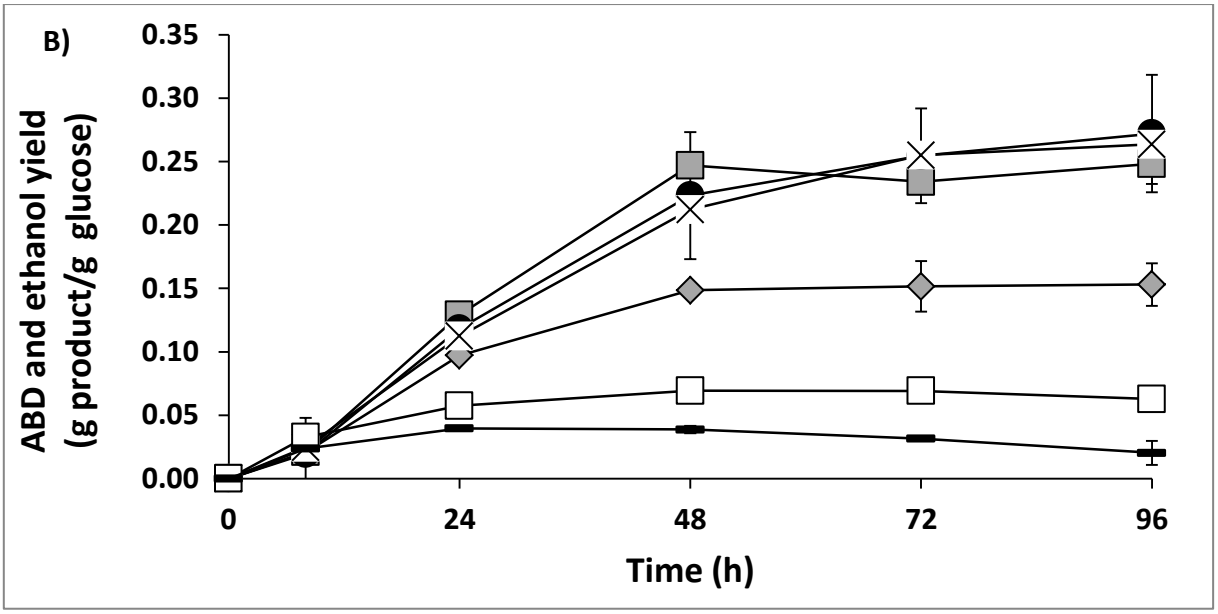


Figure 3.4: Glucose conversion (A) and ABD yield (B) in M9 culture medium supplemented with 25 g/L of glucose as a function of time in the presence of *E. coli* JFR1. Source of nitrogen: urea at 2.5 g/L of nitrogen (♦), 5.0 g/L of nitrogen (■), 7.0 g/L of nitrogen (●) and 10.0 g N/L (×). Additionally, the highest (□) and lowest (▬) ethanol yields obtained for 10.0 g N/L and 2.5 g N/L are represented respectively. Results are means ± SD of 3 replications performed in flasks at 37ºC, atmospheric pressure, initial pH of 6.5, 100 rpm and 10% v/v of inoculum in a working volume of 200 mL (batch fermentation).

The rise of ABD yield in the presence of urea when fermenting carbon sources was also reported in the presence of other bacteria. For example, the ABD yield increased by adding a nitrogen concentration of 12.3 g N/L in the presence of *K. oxytoca* ME-303 (5% (v/v) of inoculum) for 44 h at 37°C, initial pH of 6.5 and 200 rpm fermenting 60 g/L of a mixture of glucose-xylose (2:1, w/w) (Ji et al. 2009b). Wong et al. (2012) showed the improvement of ABD yield using 0.9 g N/L in the presence of *Klebsiella* sp. Zmd30 (2% (v/v) of inoculum) at 30°C and 200 rpm (initial pH and fermentation time non specified) fermenting 160 g/L of glucose; whereas a concentration of 1.8 g N/L was reported as the optimal one fermenting 160 g/L of glucose in the presence of *B. subtilis* SF4-3 (5% (v/v) of inoculum) at 37ºC and 180 rpm (pH non specified) for 96 h (Tian et al. 2014).

The concentration and the type of nitrogen source are linked to the formation of ABD. The use of urea improved the ABD yield compared to the experiments performed without an ANS. On the other hand, the use of $NaNO_3$ did not generate ABD.

3.6 Conclusion

This study was based on the glucose fermentation using a genetically modified strain of *Escherichia coli* MG1655/ALDH+*bud*ABC (*E. coli* JFR1). In a first step, the growth of ECW and *E. coli* JFR1 was followed by 2 techniques: OD_{600} and CFU. While the bacterial growth for both ECW and *E. coli* JFR1 in LB culture medium was the same according to the OD_{600}

method (near 0.8), the CFU method gave more realistic results about the bacterial growth. The population of ECW and *E. coli* JFR1 was $1.1x10^9$ and $6.5x10^8$ CFU/mL respectively, showing that the genetic modification of bacteria influences their growth decreasing the bacterial population, maybe due to the introduced gene toxicity.

The cheapest tested culture medium, M9, was selected to produce ABD (acetoin and 2,3-butanediol). The fermentation of 12.5 and 25 g/L of glucose gave the lowest ABD yields (0.18 and 0.14 g ABD/g glucose, respectively) using MOPS, the most expensive culture medium.

The addition of urea to M9 culture medium improved the ABD yield. The highest ABD yield was 0.27 g ABD/g glucose using 7.0 g N/L at 96 h in the presence of 25 g/L of glucose. The use of sodium nitrate prevented the ABD formation.

Depending on the source, concentration and even the combination of different compounds present in the culture medium, the production of ABD could be affected, maybe in a critical way such as with $NaNO_3$. In addition, it would be interesting and highly recommended to carry out an estimation of the bacterial population before performing fermentations since, as shown the results of ECW ($1.1x10^9$ CFU/mL) and *E. coli* JFR1 ($6.5x10^8$ CFU/mL), the number of bacteria (the inoculum) changes from a strain to another.

3.7 Acknowledgement

Michèle Heitz is grateful to Fond Québécois de la Recherche sur la Nature et les Technologies (FRQNT) in partnership with MAPAQ-NOVALAIT (Innovation en Production et en Transformation Laitières) for the grant attributed to the research program "Développement d'une biotechnologie verte innovatrice pour valoriser des résidus fromagers". The authors are grateful to the laboratory technicians (Department of chemical and biotechnological engineering of Université de Sherbrooke) Isabelle Arsenault and Valérie Larouche for their support and help. The authors would like to particularly thank Pr. Sébastien Rodrigue and the master student Jean-François Rousseau (Department of biology of Université de Sherbrooke) for providing the genetically modified strain *E. coli* JFR1.

Chapter 4. Fermentation of whey and its permeate using a genetically modified strain of *Escherichia coli* to produce 2,3-butanediol

Avant-propos:

L'article "Fermentation of whey and its permeate using a genetically modified strain of *Escherichia coli* to produce 2,3-butanediol" a été soumis dans un journal scientifique international (Environmental Science and Pollution Research) le 10 juillet 2018. Un autre article relatif au chapitre 4 (partie 2) devrait être soumis ultérieurement dans un Journal scientifique international. Une autorisation pour publication de la 2^{e} partie du chapitre 4 doit être demandée au préalable à Novalait. La version de l'article présenté dans le document diffère de ce qui a été soumis.

TITRE: Fermentation du lactosérum et de son perméat en présence d'une souche génétiquement modifiée d'*Escherichia coli* afin de produire du 2,3-butanediol

Title: Fermentation of whey and its permeate using a genetically modified strain of *Escherichia coli* to produce 2,3-butanediol.

David Fernández-Gutiérrez [a,c], Marc Veillette [a], Antonio Avalos Ramirez [b], Anne Giroir-Fendler [c], Nathalie Faucheux [a] and Michèle Heitz [a, d]

[a] Université de Sherbrooke, Department of Chemical Engineering and Biotechnological Engineering. Faculty of engineering, 2500 boul. de l'Université, Sherbrooke, (Québec), Canada, J1K 2R1

[b] Centre National en Électrochimie et en Technologies Environnementales, Shawinigan, (Québec), Canada, G9N 6V8

[c] Université Lyon 1, CNRS, UMR 5256, Institut de Recherche sur la Catalyse et l'Environnement de Lyon (IRCELYON), 2 avenue Albert Einstein, 69626 Villeurbanne, France

[d] Corresponding author: E-mail address: Michele.Heitz@USherbrooke.ca

Contribution to the document: This paper is the relevant for the project since the main objective is to ferment the lactose contained in the whey (W) and permeate whey (PW) by an improved genetically modified strain of *Escherichia coli* (*E. coli* JFR12) in order to produce 2,3-butanediol (2,3-BD). In addition, different operational parameters (dilution ratio with M9 culture medium, inoculum size, initial pH and agitation rate) were tested in order to study their influence on 2,3-BD yield. Moreover, a scaling up of the fermentation volume (4 times) was performed in a 2 L bioreactor besides the injection of air (2 L/min) in order to improve the formation of 2,3-BD. The potential of *E. coli* JFR12 to valorize W and PW was shown.

Fermentation of whey and its permeate using a genetically modified strain of *Escherichia coli* to produce 2,3-butanediol

4.1 Résumé

Le lactosérum (W) est obtenu lors de la fabrication du fromage et le perméat de lactosérum (PW) est obtenu après la séparation des protéines du W. Les deux effluents contiennent du lactose, source de carbone, et ils peuvent affecter l'environnement s'ils sont rejetés dans la nature sans traitement préalable. L'objectif de ce travail de recherche a été d'étudier la transformation du lactose par fermentation en 2,3-butanediol (2,3-BD) en présence d'une souche d'*Escherichia coli* K12 MG1655 (*E. coli* JFR12) génétiquement modifiée afin d'évaluer le potentiel du W et PW comme sources de lactose. Le lactose (un disaccharide), le glucose et le galactose (monosaccharides présents dans le lactose) sous des concentrations de 12.5, 25 et 50 g/L ont été fermentés dans le milieu de culture synthétique (milieu M9) inoculé à 10% (v/v) d'inoculum, 37ºC, 1 atm, initial pH 7.4 et 100 rpm pendant 72 h dans des fioles de 0.5 L (volume de travail de 0.2 L). Dans ces conditions opératoires, le meilleur rendement en 2,3-BD a été de 0.38 g/g de saccharide en présence de 25 g/L de glucose ou de lactose à 48 et 72 h, respectivement, et de 0.19 g/g de galactose à 48 h. Donc, *E. coli* JFR12 peut transformer le galactose en 2,3-BD mais le rendement en 2,3-BD est deux fois plus faible si on le compare avec celui issu du glucose à 48 h. La fermentation du W (31 g/L) ou du PW (34 g/L) dilués avec le milieu M9 (50:50, 75:25 et 100:0, v/v) a été réalisée sous les mêmes conditions opératoires. La fermentation du W et du PW en absence de M9 (100:0, v/v) a fourni les rendements les plus élevés en 2,3-BD (jusqu'à 0.43 g/g lactose) à 72 h. Tandis que le rendement le plus élevé en acétoïne (0.12 g/g lactose) a été obtenu avec une dilution de 50:50 (v/v) du W ou du PW avec M9 à 72 h. L'effet de trois paramètres (pH initial (de 6.5 à 7.4), quantité d'inoculum (de 5 à 10%, v/v) et vitesse d'agitation (de 50 à 200 rpm) a été testé pour étudier l'influence de ces paramètres sur le rendement en 2,3-BD en absence de M9. Le pH initial n'affecte pas le rendement en 2,3-BD (0.45 et 0.42 g/g lactose pour le W et le PW, respectivement). L'utilisation de 5% (v/v) d'inoculum a permis d'obtenir un rendement en 2,3-BD légèrement inférieur par comparaison avec les expériences réalisées à 10% (v/v) d'inoculum. Les résultats obtenus en faisant varier la vitesse d'agitation ont montré que le rendement en 2,3-BD a diminué et que celui de l'acétoïne a augmenté avec une vitesse d'agitation de 200 rpm. Ce phénomène peut s'expliquer par une augmentation de l'oxygène transféré au milieu de culture sous une agitation de 200 rpm en comparaison avec une agitation de 100 rpm. De plus, des fermentations du W et du PW dans un bioréacteur de 2 L ont été effectuées en conditions anaérobies et micro-aérobies pour évaluer l'augmentation du volume réactionnel sur le rendement en 2,3-BD. Les conditions anaérobies ont conduit à des rendements en 2,3-BD plus faibles, environ 0.20 g/g de lactose lors de la fermentation du W à 72 h, tandis que l'ajout d'air (2 L/min) a permis l'obtention d'un rendement en 2,3-BD similaire à celui obtenu dans des fioles de 0.5 L, 0.40 g/g de lactose après 24 h en utilisant du W à 10% (v/v) d'inoculum, 37ºC, 1 atm, pH initial de 7.4 et vitesse d'agitation de 100 rpm. Les résultats ont montré que *E. coli* JFR12 est aussi efficace que les producteurs naturels de 2,3-BD pour fermenter le lactose et obtenir du 2,3-BD.

Mots-clefs: Lactose, fabrication de fromage, glucose, galactose, fermentation, 2,3-butanediol, acétoïne, Escherichia coli.

4.2 Abstract

Whey (W) is generated during cheese manufacturing and permeate whey (PW) is obtained after W deproteinization. As both effluents contain lactose, a carbon source, and they can cause environmental issues if they are released in the environment without a treatment. The aim of the present study was to test the transformation of lactose via fermentation into 2,3-butanediol (2,3-BD) in the presence of a genetically modified strain of *Escherichia coli* K12 MG1655 (*E. coli* JFR12) in order to evaluate the potential of W and PW as lactose sources. Lactose (a disaccharide), glucose and galactose (monosaccharides of lactose) at concentrations of 12.5, 25 and 50 g/L were fermented in a synthetic culture medium (medium 9, M9) inoculated at a ratio of 10% (v/v), at 37ºC, 1 atm, initial pH 7.4 and 100 rpm for 72 h in flasks of 0.5 L with a working volume of 0.2 L. Under these operating conditions, the highest 2,3-BD yield was around 0.38 g/g saccharide in the presence of 25 g/L of glucose or lactose at 48 and 72 h, respectively, and 0.19 g/g galactose at 48 h. Therefore, *E. coli* JFR12 could transform galactose into 2,3-BD but being its yield the half of one observed with glucose at 48 h. Whey (31 g/L lactose) or PW (34 g/L lactose) were fermented in dilution with M9 (50:50, 75:25 and 100:0, v/v) under the same operating conditions. The fermentation of W and PW in absence of M9 (100:0, v/v) produced the highest 2,3-BD yield (up to 0.43 g/g lactose) at 72 h. Whereas, the highest acetoin (A) yield of 0.12 g/g lactose was obtained with the dilution of W and PW (50:50, v/v) in M9 at 72 h. The effect of three operating parameters: initial pH (from 6.5 to 7.4), inoculum size (from 5 to 10%, v/v) and agitation rate (from 50 to 200 rpm) on the 2,3-BD yield was studied in the presence of undiluted W and PW. The initial pH did not affected the 2,3-BD yield (0.45 and 0.42 g/g lactose for W and PW, respectively). The use of 5% (v/v) of inoculum size gave a slightly lower 2,3-BD yield compared to 10% (v/v) inoculum. The results obtained testing different agitation rates showed that the 2,3-BD yield decreased at a high agitation rate (200 rpm); whereas the A yield increased. This phenomenon may be due to a higher transfer of oxygen to the culture medium at 200 rpm compared with an agitation rate of 100 rpm. Moreover, W and PW were fermented in a 2 L bioreactor under anaerobic and micro-aerobic conditions in order to test the scale-up on the 2,3-BD yield. Under anaerobic conditions, the 2,3-BD yield was near 0.20 g/g lactose at 72 h using W; whereas the aeration (2 L/min of air) permitted to obtain a 2,3-BD yield similar to fermentations in 0.5 L flask, 0.40 g/g lactose after only 24 h at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm. The results show that *E. coli* JFR12 is as efficient as natural 2,3-BD producers fermenting lactose in order to obtain 2,3-BD with the advantage of being an innocuous strain.

Keywords: Lactose, cheese manufacturing, glucose, galactose, fermentation, 2,3-butanediol, acetoin, Escherichia coli.

4.3 Introduction

Nowadays, the worldwide manufacturing of the dairy products (e.g. cheese) has increased. For instance, the worldwide production of cheese increased by 25% between 2004 and 2014 to reach 22.6 million tons (FAO-FAOSTAT, 2017d). Cheese is produced by coagulating the casein present in milk, by the action of enzymes like chymosin or acids like citric acid

(Panesar et al. 2007). Nine (9) L of whey (W) per kg of cheese are generated during the cheese making process (Guimaraes et al. 2008). Taking into account the amount of cheese produced worldwide in 2014, the volume of W generated can be estimated around 203 million m^3. Whey can be valorized by ultrafiltration (membranes) in order to obtain whey proteins, which are used in food as emulsifier or as gelling and water binding agent (Rebouillat and Ortega-Requena 2015), generating another effluent: the permeate whey (PW). Whey contains up to 54 g/L of lactose (a disaccharide composed of glucose and galactose), whereas PW has a lactose concentration higher than 80 g/L (Panesar et al. 2007, Smith et al. 2016). Such lactose content represents high biological and chemical oxygen demands (BOD and COD, respectively). The BOD and COD contents are similar for W and PW at around 47 and 70 g/L, respectively (Belhamidi et al. 2015). When these effluents are released in rivers or lakes without treatment, they cause an oxygen reduction perturbing the aquatic life (De Jesus et al. 2015). Both W and PW can be valorized by means of lactose fermentation to produce, for example biogas, hydrogen (H_2), acetoin (A) and alcohols such as 2,3-butanediol (2,3-BD) (Antonopoulou et al. 2008, Becerra et al. 2015, Parra Huertas 2009).

The diol, 2,3-butanediol (2,3-BD), is a valuable compound highly used in cosmetic, pharmaceutical and petrochemical industries (Ji et al. 2009b). For example, 2,3-BD is involved in the manufacture of perfumes, and can be used as an antifreeze agent (due to its normal melting point of -60ºC) (Celińska and Grajek 2009, Garg and Jain 1995, Ji et al. 2009b, Mazumdar et al. 2013). 2,3-butanediol is a chemical platform to produce other chemicals, such 1,3-butadiene, used for synthetic rubber manufacturing, and methyl ethyl ketone, a solvent and fuel additive and polyurethane methacrylate, used in cardiovascular applications (Cherng et al. 2013, Wong et al. 2014). Acetoin, the 2,3-BD precursor via a biological process, is an interesting compound since it is widely used in cosmetics as perfume and lotion additive due to its sweet fragrance, as well as in food industry as an additive due to its buttery flavor (Dai et al. 2015, Ji et al. 2009b, Xiao and Lu 2014).

2,3-butanediol (2,3-BD) can also be obtained from A by its hydrogenation using a heterogeneous catalyst based on Ru at temperature and hydrogen partial pressures up to 175ºC and almost 20 atm, respectively, for 16 h (Ochoa Gómez et al. 2017). Bacteria species like *Bacillus*, *Enterobacter* and *Klebsiella* are natural producers of 2,3-BD at high yields using lactose contained in W and PW (Guo et al. 2017). For example, Guo et al. (2017) reported a 2,3-BD yield of 0.43 g/g lactose using *K. pneumoniae* CICC 10781 fermenting 74 g/L of lactose with an inoculum of 10% (v/v) at 35ºC, pH 6, 300 rpm and 1 vvm after 32 h. However, some of these bacteria such as *Enterobacter* and *Klebsiella* species are pathogen, limiting their use in the industry (Cho et al. 2015, PHAC 2017). Thus, it is necessary to modify genetically non-pathogen strains species such as *Escherichia coli* K12 MG1655 to produce 2,3-BD since they do not possess its metabolic pathway (Bialkowska 2016, Fernández-Gutierrez et al. 2017). Hence, in the current study, *E. coli* K12 MG1655, an innocuous strain to human being (iGEM 2017), was considered to host the 2,3-BD metabolic pathway from *Enterobacter cloacae* (named as *E. coli* JFR12) in order to ferment both W and PW and produce 2,3-BD.

The main objective of this study was to ferment W and PW to produce 2,3-BD using *E. coli* JFR12. In addition, to the best of our knowledge, neither W or PW have been valorized into 2,3-BD using an *E. coli* strain.

Previously, M9 supplemented with 15 g/L of urea was determined as the most suitable culture medium to produce ABD (A + 2,3-BD) from glucose using the modified strain *E. coli* JFR1 (Fernández-Gutierrez et al. 2018). In the present study, the effect of glucose, galactose and lactose concentrations in M9 fermented with *E. coli* JFR12 was studied. In addition, two mixtures of glucose-galactose (1:1, w/w) in M9 for a total concentration of 25 and 50 g/L were fermented. Afterwards, two natural industrial effluents, W1 and PW1, were fermented in order to test the ability of *E. coli* JFR12 to grow and transform the lactose in W1 and PW1 into 2,3-BD at different dilution ratios with M9. The effects of initial pH, inoculum size and agitation on 2,3-BD yield were also studied in absence of M9. Moreover, 'second' batches of W (W2) (51 g lactose/L) and PW (PW2) (47 g lactose/L) were fermented in flasks in order to test the influence of a higher lactose concentration in both effluents. Finally, W2 and PW2 were studied in a 2 L bioreactor (0.8 L of working volume) to compare the 2,3-BD yields with those obtained in 0.5 L flask (0.2 L of working volume). The effect of the aeration on 2,3-BD and A yields was also performed fermenting only W2.

4.4 Materials and methods

4.4.1 Microorganism

The microorganism used in the present study was *E. coli* K12 MG1655, hosting the 2,3-BD metabolic pathway from *Enterobacter cloacae* (*E. coli* JFR12). The biosynthetic pathways of phosphate acetyltranferase (*pta*), fumarate reductase (*frd*) and fermentative D-lactate dehydrogenase (*ldhA*) were blocked to avoid the formation of acetic acid, succinic acid and lactic acid, respectively. The modified strain was *E. coli* K12 MG1655 pir + D*pta*, D*ldhA*, D*frd* + *bud*ABC (integrated in the chromosome) + plasmid pOSIP-TT + *bud*ABC (not integrated in the chromosome). The conservation of *E. coli* JFR12 was performed at -81ºC in a blend (50:50, v/v) of glycerol and lysogeny broth (LB) culture medium.

4.4.2 Culture media

Lysogeny broth (LB) culture medium was used to grow *E. coli* JFR12 (seed culture). LB culture medium has proved to be suitable for recombinant *E. coli* strains (Maniatis et al. 2001). The composition of LB culture medium was prepared as follows: 10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of sodium chloride (NaCl) and distilled water (Berney et al. 2006). Medium 9 (M9) was made as follows: 12.8 g/L of sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), 3 g/L of potassium dihydrogen phosphate (KH_2PO_4), 1 g/L of ammonium chloride (NH_4Cl), 0.5 g/L of NaCl, 15 g/L of urea ($(NH_2)_2CO$), 0.49 g/L of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and 0.01 g/L of calcium chloride ($CaCl_2$) and distilled water (Fernández-Gutierrez et al. 2018).

All samples of W and PW were supplied by a local dairy industry (Lactancia Parmalat, Victoriaville, Quebec, Canada). The lactose content of W1 was 31.0 ± 0.3 g/L and of PW1 was 34.0 ± 0.3 g/L. A second batch of whey (W2) with 51 ± 2 g/L of lactose and permeate whey (PW2) with 47 ± 1 g/L of lactose were used for fermentation in a 2 L bioreactor. All industrial samples were supplemented with urea, $MgSO_4 \cdot 7H_2O$ and $CaCl_2$.

Tetracycline was added at a concentration of 15 μg/mL in order to favor the growth of the modified strain which contained the resistance plasmid, avoiding the biological contamination from other bacteria (SLH 2017).

The seed culture medium was prepared as follows: a sample of *E. coli* JFR12 from the conservation blend was taken with a tip and mixed with 10 mL of fresh LB medium in a test tube (15 mL). The test tube was incubated overnight at 37ºC (pre-culture medium). Then, 2 mL of the pre-culture medium were transferred into a 0.5 L Erlenmeyer flask containing 0.2 L of fresh LB medium and incubated in a rotary shaker incubator (Fermentation Design inc, Allentown, PA) at 37ºC, 1 atm, initial pH 6.5 and 100 rpm to reach a bacterial population of $4.8x10^7$ colony-forming unit (CFU)/mL in 9 h. The seed culture medium was used to inoculate the culture media M9 and all industrial samples (diluted and undiluted with M9 or distilled water) at a ratio of 10% (v/v).

4.4.3 Fermentation parameters

The fermentations in 0.5 L flasks were performed using a working volume 0.2 L: glucose, galactose and lactose at initial concentration of 12.5, 25 and 50 g/L, mixture of glucose-galactose (1:1, w/w) at initial concentrations of 25 and 50 g/L, W1 and PW1 diluted with M9 (50:50, 75:25 and 100:0, v/v), and W2 and PW2 diluted with distilled water (dilution at 30 and 18%, v/v, respectively). The flasks were inoculated with a seed culture at a ratio of 10% (v/v) and incubated at 37ºC, 1 atm, initial pH 7.4 and 100 rpm for 72 h. In order to analyze the effect of fermentation operating conditions, W1 and PW1 were operated at: i) initial pHs of 6.5, 7.0 and 7.4, 10% (v/v) of inoculum, 37ºC, 1 atm and 100 rpm; ii) inoculum sizes of 5, 7.5 and 10% (v/v), 37ºC, 1 atm, pH 7.4 and 100 rpm; and iii) agitation rates of 50, 100 and 200 rpm, 10% (v/v) of inoculum, 37ºC, 1 atm and an initial pH of 7.4.

Whey (W1 and W2) and permeate whey (PW1 and PW2) were fermented using a 2 L bioreactor (0.8 L of working volume) at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm for 72 h. The effect of air addition was tested using the 2 L bioreactor at 2.5 vvm fermenting diluted W2. All the operating conditions are summarized in Table 1.

4.4.4 Analytical methods

The concentration of saccharides (glucose, galactose and lactose), 2,3-BD, A and ethanol (Et) was determined by high performance liquid chromatography (HPLC) using an Agilent series 1100 chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a column Aminex HPX-87H (300 x 7.8 mm) at a temperature of 25ºC; sulfuric acid 0.005M was used as a mobile phase (0.4 mL/min). Saccharides, 2,3-BD and Et were quantified by a Refractive index (RID) detector, and A by an ultraviolet (UVD) detector.

The reagents used for the analytical method were HPLC grade: Glucose (≥ 99.5%), 2,3-BD (98%) and A were purchased at Sigma-Aldrich (Canada), galactose (> 99%) was bought at Acros Organics (Belgium), lactose (≥ 99%) and Et (≥ 99%) were purchased at Fisher Scientific Inc. (Canada).

4.4.5 Statistical analysis

The influence of operating parameters (i.e., saccharide concentration, mixture ratios with M9 (W1 and PW1) and with distilled water (W2 and PW2), inoculum size, initial pH, agitation rate and anaerobic conditions) on 2,3-BD yield were determined by means of an analysis of variance (ANOVA) with a P-value lower than 0.05. Dixon's Q test was used to estimate and reject outlier values of fermentative products yield (2,3-BD, A and Et) using a confidence level of 95% (Rorabacher 1991).

Table 4.1: Operating conditions used to ferment saccharides (glucose, galactose, lactose and the mixture glucose-galactose, G-Gal) in M9, whey (W1) and permeate whey (PW1) diluted with M9, and the study of inoculum size, initial pH and agitation in flask. New batch of W (W2) and PW (PW2) diluted and undiluted were tested in flask and in a 2 L bioreactor. Fermentations performed at 37ºC, 1 atm and 72 h.

Parameter	Carbon source and concentration (g/L)	Culture medium	Inoculum size (%, v/v)	Initial pH	Agitation (rpm)
Kind and concentration of saccharide[1]	Glucose, galactose and lactose: 12.5, 25 and 50 G-Gal: 25 and 50	M9	10	7.4	100
Dilution of W1[1]	Lactose: 15.5	W1:M9 (50:50)	10	7.4	100
	Lactose: 23	W1:M9 (75:25)			
	Lactose: 31	W1:M9 (100:0)			
Dilution of PW1[1]	Lactose: 17	PW1:M9 (50:50)	10	7.4	100
	Lactose: 25.5	PW1:M9 (75:25)			
	Lactose: 34	PW1:M9 (100:0)			
Inoculum size[1]	Lactose: 31 for W Lactose: 34 for PW	W1 PW1	5	7.4	100
			7.5		
			10		
Initial pH[1]	Lactose: 31 for W Lactose: 34 for PW	W1 PW1	10	6.5	100
				7.0	
				7.4	
Agitation[1]	Lactose: 31 for W Lactose: 34 for PW	W1 PW1	10	7.4	50
					100
					200
Scale-up[2]	Lactose: 31 and 51	W2 (diluted and undiluted)	10	7.4	100
	Lactose: 34 and 47	PW2 (diluted and undiluted)	10	7.4	100
	Lactose: 31	W2 (diluted)[3]	10	7.4	100

***All culture media were supplemented with urea (15 g/L), $MgSO_4 \cdot 7H_2O$ (0.49 g/L) and $CaCl_2$ (0.01 g/L).**

[1] Experiments performed in flask (0.2 L of working volume)

[2] Experiments performed in bioreactor (0.8 L of working volume)

[3] Air addition at 2.5 vvm

4.5 Results and discussion

4.5.1 Effect of kind and concentration saccharides on 2,3-butanediol yield

The modified *E. coli* JFR12 was used to produce 2,3-BD by fermentation of saccharides (lactose, glucose and galactose) and industrial wastes (W1 and PW1) as carbon sources. Figure 4.1 shows the metabolic pathway to produce 2,3-BD from lactose using a genetically modified strain of *E. coli* K12 MG1655 (*E. coli* JFR12). The disaccharide has to be first hydrolyzed into glucose and galactose (monosaccharides) by the enzyme β-galactosidase, breaking the glycosidic bond. Glucose is enzymatically fermented and transformed into pyruvic acid (PA) by glycolysis and galactose is transformed by the Leloir pathway into glucose-6-phosphate, which is transformed into PA by glycolysis (Lazar et al. 2015). Then, 3 successive enzymatic reactions are performed: i) from PA to α-acetolactate by the enzyme α-acetolactate synthase (ALS), ii) from α-acetolactate to A by the enzyme α-acetolactate decarboxylase (ALDC), and iii) from A to 2,3-BD by the enzyme 2,3-butanediol dehydrogenase (BDH) (Xiao and Lu 2014, Xu et al. 2014, Xu et al. 2015). Under aerobic conditions, which may inhibit the ALS enzyme, α-acetolactate may be transformed into diacetyl; and then into A, being A transformed into 2,3-BD (Shi et al. 2014). In these reactions, the coenzyme nicotinamide adenine dinucleotide (NAD), which has a reduced and an oxidized form ($NADH + H^+$ and NAD^+, respectively), is involved in the transformation of diacetyl into A and of A into 2,3-BD where NADH is oxidized to NAD^+ (Xu et al. 2014). The fermentation conditions to perform this series of experiments are shown in Table 4.1.

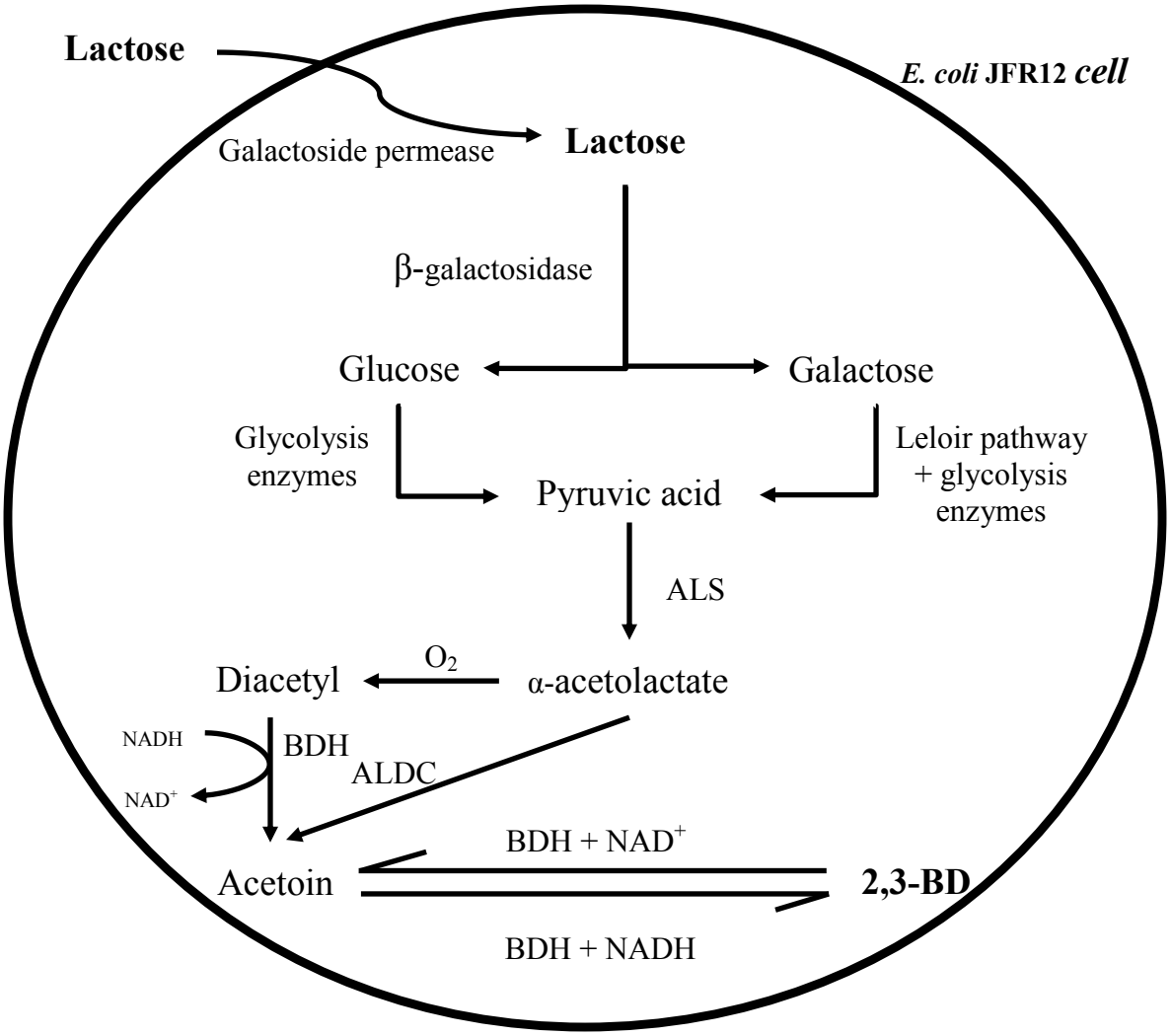


Figure 4.1: Metabolic pathway to produce 2,3-BD from lactose fermentation in the presence of a genetically modified strain of Escherichia coli. α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC) and 2,3-butanediol dehydrogenase (BDH). Reduced NADH) and oxidized (NAD+) form of nicotinamide adenine dinucleotide. Sources: Lazar et al. (2015), Mazumdar et al. (2013), Xiao and Lu (2014), Xu et al. (2014), Xu et al. (2015).

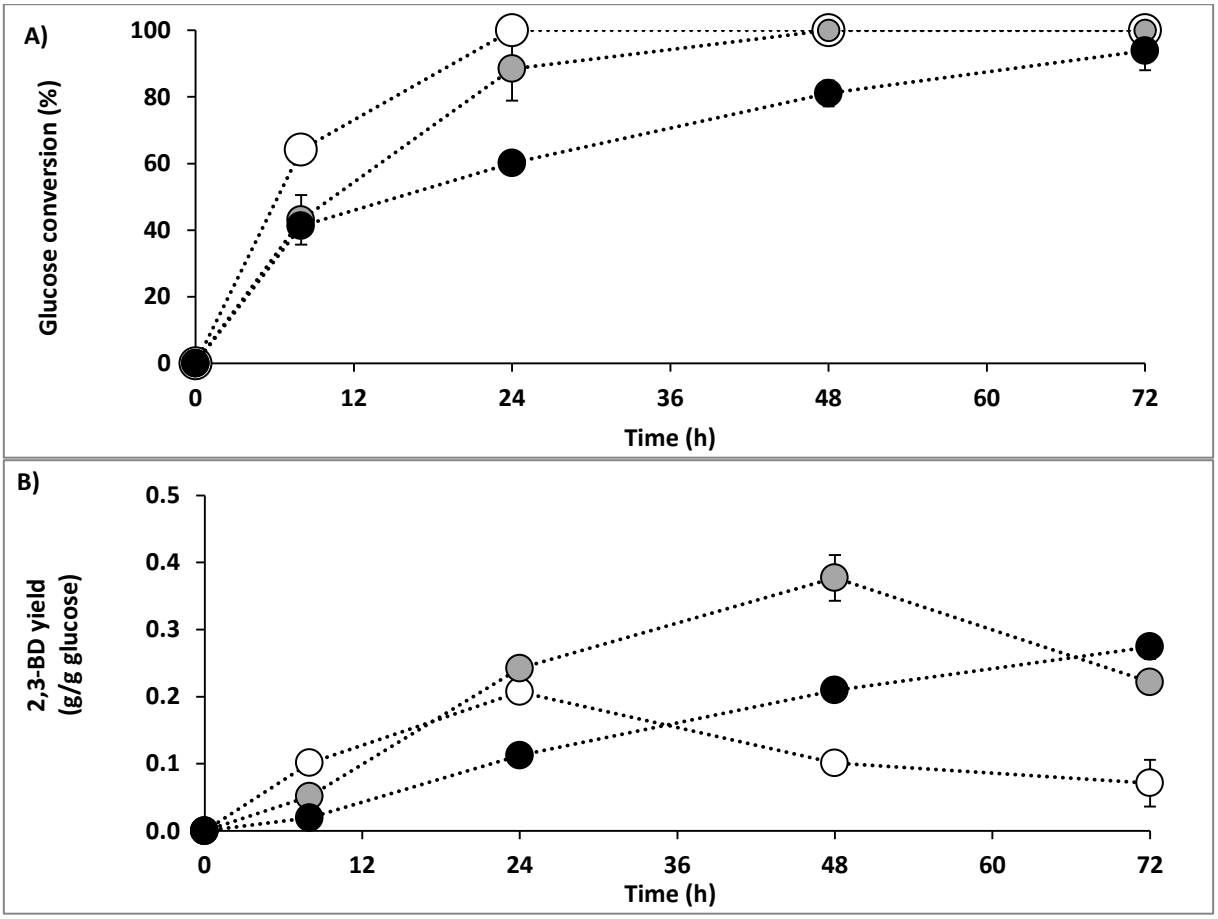
4.5.1.1 Glucose

Figure 4.2 (A, B and C) shows the glucose conversion, 2,3-BD and A yields for 3 initial glucose concentrations ($[Glu]_0$ = 12.5, 25 and 50 g/L) as a function of time. After the first 8 h, the conversion of glucose was around 60% for the $[Glu]_0$ of 12.5 g/L and 40% for the $[Glu]_0$ of 25 and 50 g/L. Glucose conversion was 100% at 24 h for a $[Glu]_0$ of 12.5 g/L, and at 48 h for a $[Glu]_0$ of 25 g/L. However, for a $[Glu]_0$ of 50 g/L, the conversions were close to 60 and 80% at 24 and 48 h, respectively; whereas the maximum conversion was slightly lower compared to 12.5 and 25 g/L of glucose and reached 94% after 72 h (Figure 4.2A). For the 3

$[Glu]_0$, at 8 h of fermentation, glucose conversions varied between 40 and 60%, which might be due to the proliferation of bacterial cells (Chan et al. 2016). The bacterial population might be affected by a high substrate concentration, leading to a slower bacterial growth (Chan et al. 2016). This may explain the glucose conversions observed for $[Glu]_0$ of 25 and 50 g/L, where longer times (48 and 72 h, respectively) were required in order to obtain the maximum conversion.

For the $[Glu]_0$ of 12.5 g/L and 25 g/L, the 2,3-BD reached a maximum yield and then decreased. For 12.5 g/L, the 2,3 BD yield was 0.21 g/g glucose at 24 h and then decreased to 0.07 g/g glucose at 72 h; for 25 g/L it increased up to 0.38 g/g glucose at 48 h, and then decreased to 0.22 g/g glucose at 72 h ($p < 0.05$). In the case of $[Glu]_0$ of 50 g/L, the 2,3-BD yield increased up to 0.27 g/g glucose ($p < 0.05$) up to 72 h (Figure 4.2B). In this way, the maximum 2,3-BD yield increased from a $[Glu]_0$ of 12.5 to 25 g/L and decreased in the range from 25 to 50 g/L of glucose, which points out that a high substrate concentration not only may have an inhibitory effect on the bacterial growth, but also may affect the 2,3-BD formation, as suggested by other authors (Chan et al. 2016, Priya et al. 2016, Xin et al. 2016, Yang et al. 2011).

The accumulation of A decreased when $[Glu]_0$ increased ($p < 0.05$). At 72 h for 12.5 g/L of glucose, the A yield increased up to 0.18 g/g glucose; whereas for 25 g/L, the maximum yield was 0.08 g/g glucose, and for 50 g/L, A was not detected (Figure 4.2C).



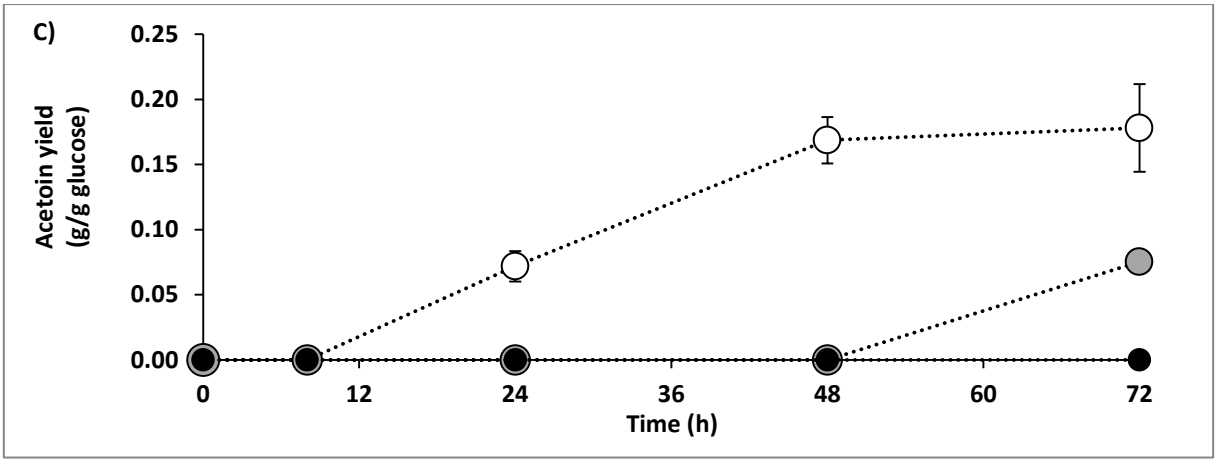


Figure 4.2: Glucose conversion (Figure 4.2A), 2,3-BD (Figure 4.2B) and A (Figure 4.2C) yields as a function of time in the presence of *E. coli* JFR12 at 3 $[Glu]_0$. Glucose concentration: 12.5 (○); 25 (●) and 50 (●) g/L. Results are means ± SD of 2 experiments performed in duplicate.

During the conversion of glucose to PA, 2 molecules of NAD^+ are transformed into NADH. On the other hand, when A is transformed into 2,3-BD by the enzyme BDH, a molecule of NADH is oxidized to NAD^+. Therefore, BDH enzyme is regulated to produce A or 2,3-BD by the coenzymes NADH and NAD^+ (Figure 1). These transformations occur when glucose is available in the culture medium. However, when glucose is exhausted, the pool of free NAD^+ must be higher than NADH since NAD^+ cannot be reduced to NADH in the PA metabolic pathway; while NADH is oxidized to NAD^+ during the reaction from A to 2,3-BD. This causes the reversible reaction and transformation of 2,3-BD into A (Xu et al. 2014), being 2,3-BD considered as an energy-storing metabolite. This phenomenon was clearly observed in the present study for the lowest $[Glu]_0$ of 12.5 g/L between 24 and 72 h and for $[Glu]_0$ of 25 g/L between 48 and 72 h. It has also been reported by Li et al. (2010) using *E. coli* JM109 when glucose conversion was 100% for a $[Glu]_0$ of 60 g/L at 10% (v/v) of inoculum, 37ºC, pH 6.8 and 500 rpm for 48 h.

The Et maximum yields decreased from 0.09, to 0.05 and to 0.02 g Et/g glucose when glucose conversions were 100% for $[Glu]_0$ of 12.5, 25 and 50 g/L, respectively (Table 2). Ethanol formation, as 2,3-BD, depends on the use of NADH to be generated. The increase of the $[Glu]_0$ might have an effect on the enzymes involved in Et formation reducing its yield and thus increasing the 2,3-BD yield (Ji et al. 2010), as observed in the $[Glu]_0$ range between 12.5 and 25 g/L.

The highest 2,3-BD yield obtained with glucose at a concentration of 25 g/L was nearly 80% of the theoretical yield, i.e. 0.38 g/g glucose at 48 h. The 2,3-BD yield is close to the one reported for other *E. coli* strains using glucose concentrations higher than 25 g/L (from 50 to 80 g/L of glucose) ranging between 0.39 and 0.43 g/g glucose (Tong et al. 2016, Xu et al. 2014). For example, a $[Glu]_0$ of 50 g/L in M9 supplemented with 5 g/L of yeast extract fermented in the presence of *E. coli* BL21 gave a 2,3-BD yield of 0.43 g/g glucose at 1% (v/v) of inoculum, 37ºC and 180 rpm (pH not mentioned) for 24 h (Xu et al. 2014).

Table 4.2: Maximum Et yield fermenting glucose, galactose, lactose and a mixture (1:1, w/w) of glucose-galactose at different concentrations at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm.

Saccharide	Saccharide concentration (g/L)	Et yield (g/g saccharide)
Glucose	12.5	0.09
	25.0	0.05
	50.0	0.02
Galactose	12.5	0.08
	25.0	0.05
	50.0	0.02
Lactose	12.5	0.10
	25.0	0.05
	50.0	0.02
Glucose-galactose	25.0	0.05
	50.0	0.02

4.5.1.2 Galactose

Figure 4.3 (A, B and C) presents the galactose conversion, 2,3-BD and A yields as a function of time and initial galactose concentrations of 12.5, 25 and 50 g/L. At 8 h, the galactose conversion was around 40% whatever the $[Gal]_0$ used. However, at 24 h of fermentation, the galactose conversion depended on the $[Gal]_0$ reaching 100, 92 and 59% for 12.5, 25 and 50 g/L respectively. At 72 h, for the highest $[Gal]_0$ (50 g/L), the galactose conversion reached 90% (Figure 4.3A). The trend for galactose conversion was similar to the one of glucose (Figure 2A), being the lowest conversion obtained fermenting 50 g/L of galactose.

For the $[Gal]_0$ of 12.5 g/L, the maximum 2,3-BD yield increased up to 0.12 g/g galactose at 24 h and then decreased by 33% (0.08 g/g galactose) at 72 h; whereas with the $[Gal]_0$ of 25 g/L, the maximum 2,3-BD yield reached 0.17 g/g galactose at 24 h, remained nearly constant (0.19 g/g galactose) at 48 h and decreased to 0.11 g/g galactose at 72 h ($p < 0.05$). The highest 2,3-BD yield (0.23 g/g galactose) was obtained with 50 g/L of galactose at 72 h, which was around 2.9 and 2.1 fold higher ($p < 0.05$) than the yields obtained at 72 h with 12.5 g/L (0.08 g/g galactose) and 25 g/L (0.11 g/g galactose), respectively (Figure 4.3B).

For the $[Gal]_0$ of 12.5 g/L, A was detected at 24 h. The A yield reached a plateau at 0.08 g/g galactose at 48 h. For the $[Gal]_0$ of 25 g/L, A formation occurred at 48 h and reached a similar A yield (0.07 g/g galactose) at 72 h compared to the one obtained for a $[Gal]_0$ of 12.5 g/L. On the other hand, A was not detected for a $[Gal]_0$ 50 g/L (Figure 4.3C). The maximum Et yields for a $[Gal]_0$ of 12.5, 25 and 50 g/L were 0.08, 0.05 and 0.02 g/g galactose, respectively (Table 4.2).

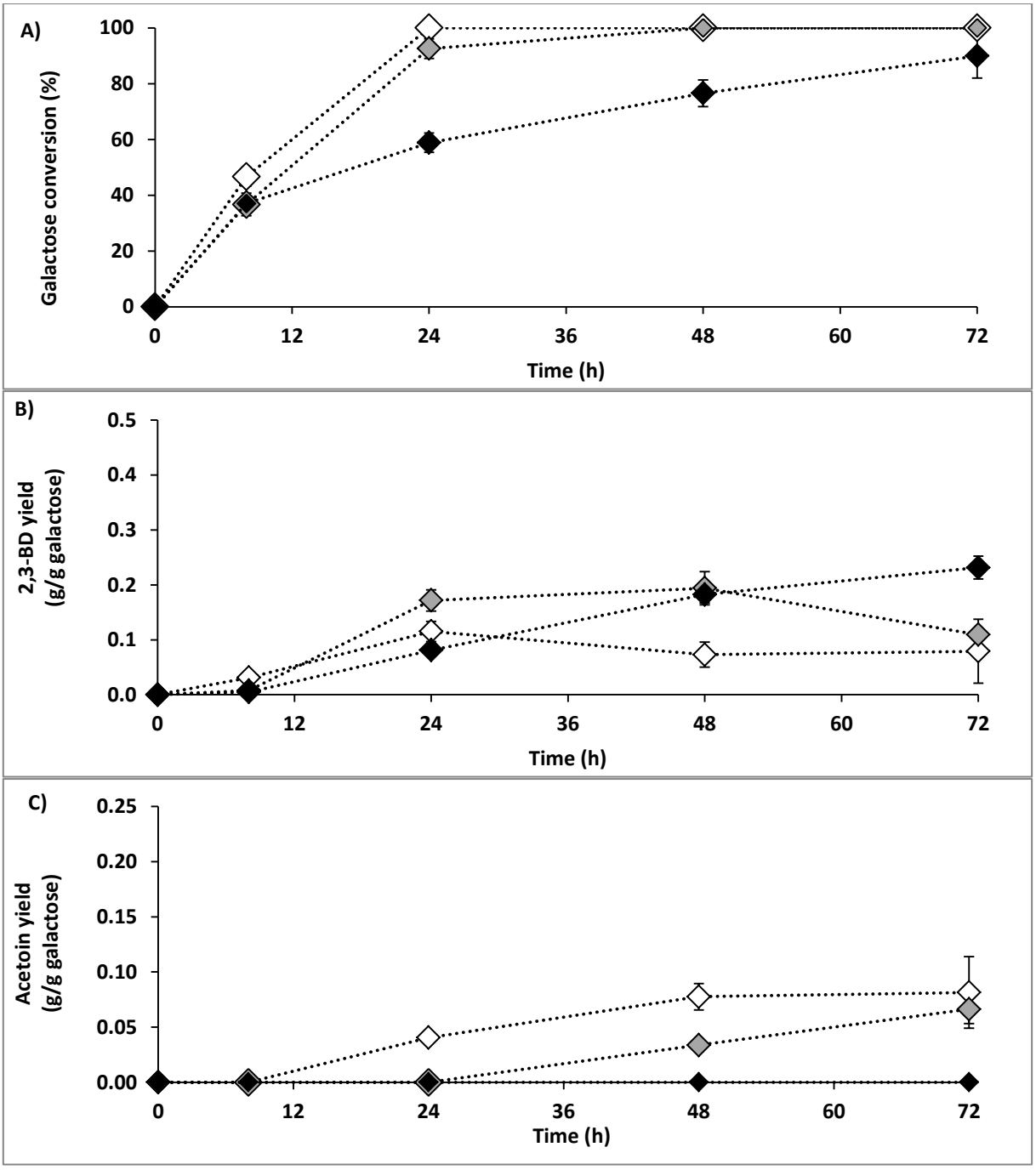


Figure 4.3: Galactose conversion (Figure 4.3A), 2,3-BD (Figure 4.3B) and A (Figure 4.3C) yields as a function of time in the presence of *E. coli* JFR12 at 3 $[Gal]_0$. Galactose concentration: 12.5 (◊); 25 (♦) and 50 (♦) g/L. Results are means ± SD of 2 experiments performed in duplicate.

According to the best of our knowledge, *E. coli* has not been previously used for galactose fermentation in order to produce 2,3-BD. However, other bacteria like *Raoultella* were used for that purpose (Kim et al. 2016). For example, using *R. ornithinolytica* B6, the 2,3-BD yield was 0.24 g/g galactose for a $[Gal]_0$ of 82 g/L (concentration calculated using the data provided

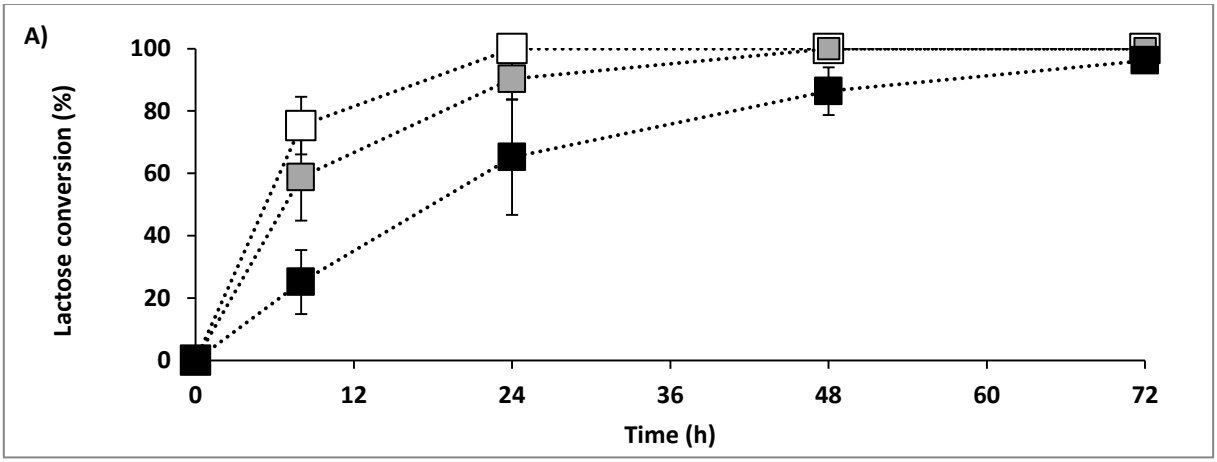
by the authors) at 10% (v/v) of inoculum, 25°C, pH 7, 200 rpm and 0.5 vvm for 36 h (Kim et al. 2016). The 2,3-BD yield was similar to the highest yield (0.23 g/g galactose) obtained in the present study with a $[Gal]_0$ of 50 g/L. As observed, *E. coli* JFR12 was able to transform galactose by fermentation into 2,3-BD but with lower 2,3-BD yields compared to those obtained with glucose. As an example, the 2,3-BD yield was twice lower at 48 h using 25 g/L of galactose and glucose: 0.19 g/g galactose and 0.38 g/g glucose, respectively.

4.5.1.3 Lactose

Figure 4.4 (A, B and C) presents the lactose conversion, 2,3-BD and A yields as a function of time and initial lactose concentrations ($[Lac]_0$ = 12.5, 25 and 50 g/L). Lactose conversion decreased with an increase of $[Lac]_0$. After 8 h of fermentation, conversion of 75, 58 and 25% were obtained for $[Lac]_0$ of 12.5, 25 and 50 g/L, respectively. A lactose conversion of 100% was achieved at 24 h for 12.5 g/L, whereas it was 90 and 65% for 25 and 50 g/L, respectively. For the $[Lac]_0$ of 25 g/L, the lactose conversion was 100% at 48 h and nearly 100% for 50 g/L at 72 h (Figure 4.4A).

Using lactose, the 2,3-BD yields were similar to glucose for the same operating conditions. For the smallest $[Lac]_0$ of 12.5 g/L, the 2,3-BD yield increased from 0.13 g/g lactose at 8 h to 0.27 g/g lactose at 24 h. Then, the 2,3-BD yield decreased by 44% (0.15 g/g lactose) at 72 h ($p < 0.05$). For the 2 other glucose concentrations, this phenomenon was not observed and the 2,3-BD yield increased with the time reaching a plateau of 0.36 g/g lactose for $[Lac]_0$ of 25 g/L at 48 h and 0.28 g/g lactose for $[Lac]_0$ of 50 g/L at 72 h (Figure 4.4B).

For $[Lac]_0$ of 12.5 g/L, A was detected at 24 h, and then its yield increased 3 fold up to 72 h: from 0.05 g/g lactose at 24 h to 0.15 g/g lactose at 72 h ($p < 0.05$). For a $[Lac]_0$ of 25 g/L, the A yield was 0.04 g/g lactose at 48 h and 0.10 g/g lactose at 72 h; whereas A was not detected for the $[Lac]_0$ of 50 g/L (Figure 4.4C). The maximum Et yields for $[Lac]_0$ of 12.5, 25 and 50 g/L were 0.10, 0.05 and 0.02 g/g lactose, respectively (Table 4.2). The Et yields were similar to the yields obtained with glucose and galactose; thus, the Et formation seems not to depend on the carbon source, but on its concentration.



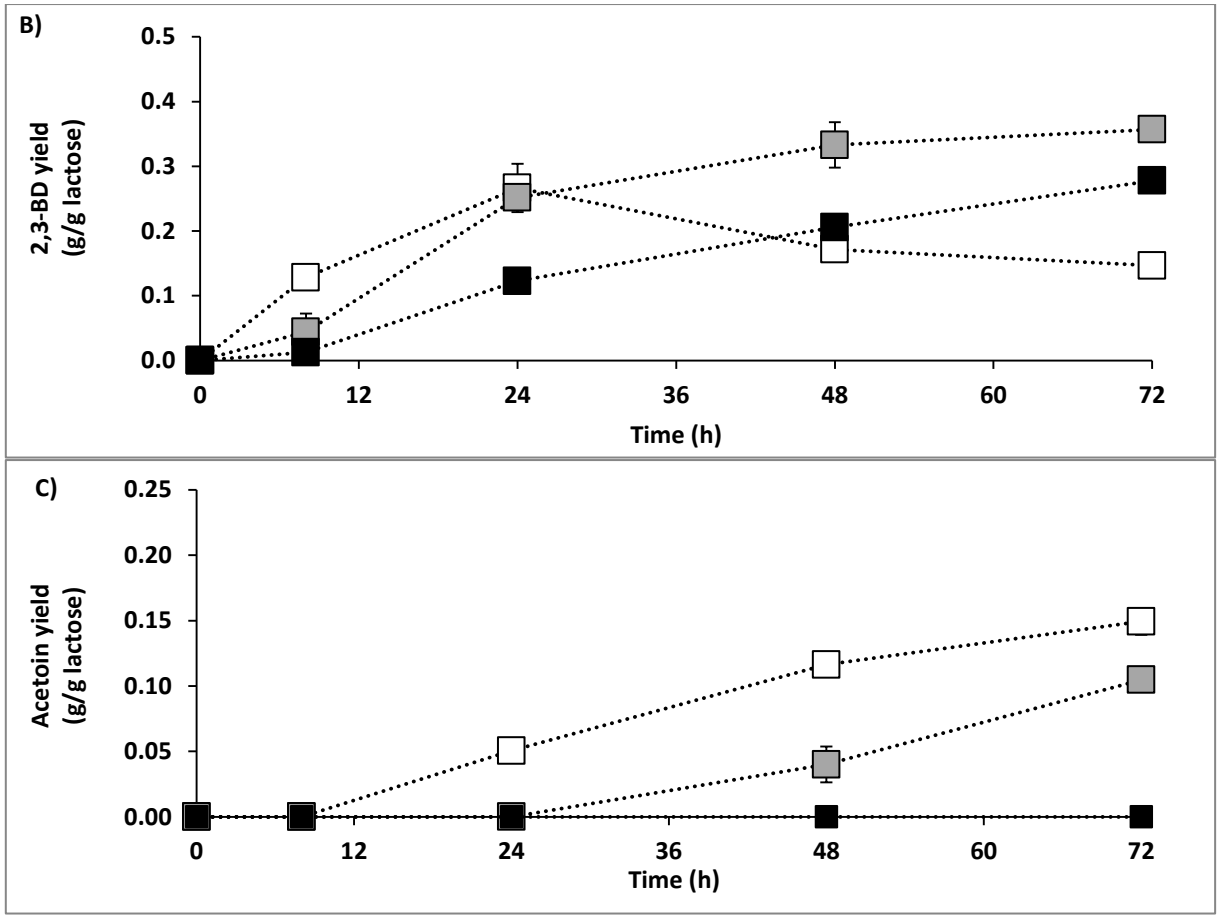


Figure 4.4: Lactose conversion (Figure 4.4A), 2,3-BD (Figure 4.4B) and A (Figure 4.4C) yields as a function of time in the presence of *E. coli* JFR12 at 3 $[Lac]_0$. Lactose concentration: 12.5 (□); 25 (■) and 50 (■) g/L. Results are means ± SD of 2 experiments performed in duplicate.

The highest 2,3-BD yield (0.36 g/g lactose) obtained in the present study from $[Lac]_0$ of 25 g/L at 72 h was similar to those obtained using other wild bacteria like *K. pneumoniae* and *K. oxytoca* (Guo et al. 2017). For example, a maximum 2,3-BD yield was reached at 24 h in the presence of *K. pneumoniae* CICC 10781 (0.38 g/g lactose) and *K. oxytoca* CICC 21518 (0.36 g/g lactose) when both strains fermented 60 g/L of lactose at 10% (v/v) of inoculum, 37ºC, initial pH 6 and 200 rpm (Guo et al. 2017). Therefore, taking into account the results obtained in the present study, *E. coli* JFR12 was able to produce 2,3-BD from lactose and, in addition, is harmless for human health.

Results show that the use of galactose as sole carbon source gave 2,3-BD yields up to 2 fold lower compared to glucose as shown in Figures 4.2B and 4.3B ($p < 0.05$). However, galactose effect on the 2,3-BD yield was not observed using lactose as a carbon source after its hydrolysis by β-galactosidase enzyme (Figure 4.4B). In this way, it can be hypothesized that β-galactosidase enzyme plays an important role on the 2,3-BD formation. In order to break down saccharides, energy (e.g. that obtained by oxidizing ATP, adenosine triphosphate) is required at the first step of the fermentation (Berg et al. 2002). According to Ishikawa et al. (2015), the β-galactosidase enzyme provides both energy and carbon when lactose is

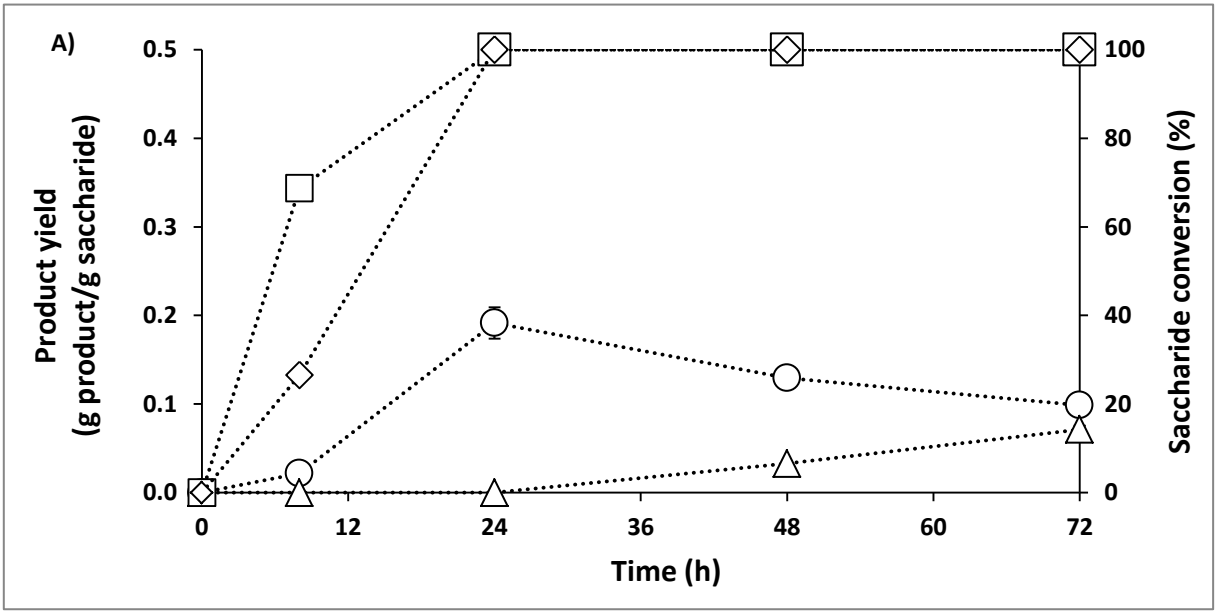
hydrolyzed, and its activity is induced in the presence of lactose (Chu et al. 2002). This may explain why lactose and glucose gave similar maximum 2,3-BD yields at 12.5, 25 and 50 g/L and why galactose alone provided lower 2,3-BD yields.

4.5.1.4 Glucose-galactose

A mixture of glucose-galactose (G-Ga) was fermented to study the effect of the mixture on 2,3-BD formation and to understand the similar 2,3-BD yields obtained for lactose and glucose. The glucose (G) and galactose (Ga) concentrations for this assay were (1:1, w/w), i.e. 12.5 and 25 g/L of each monosaccharide for a total concentration of 25 and 50 g/L.

Figure 4.5 (A and B) shows the conversion, 2,3-BD and A yields for a mixture of G-Ga (25 and 50 g/L) as a function of time. For 25 g/L of G-Ga mixture (Figure 4.5A), conversions of glucose and galactose were 70 and 27%, respectively, at 8 h, and increased to 100% for both monosaccharides after 24 h of fermentation. The 2,3-BD yield increased and reached a maximum at 24 h (0.19 g/g mixture of G-Ga); then, the 2,3-BD yield decreased by 47 % down to 0.10 g/g mixture of G-Ga at 72 h ($p < 0.05$). Acetoin was not detected until 48 h and reached a yield of 0.07 g/g mixture of G-Ga at 72 h.

For the $[G\text{-}Ga]_0$ of 50 g/L (Figure 4.5B), the conversion of glucose and galactose was 60 and 30%, respectively, at 8 h. At 24 h, the glucose conversion reached 100%, whereas the galactose conversion was 76%. The galactose conversion reached 100% at 72 h. The maximum 2,3-BD yield increased with the time and reached 0.24 g/g mixture of G-Ga at 72 h. Acetoin was not detected during the mixture of monosaccharides fermentation at 50 g/L. The maximum Et yields were 0.05 and 0.02 g/g mixture of G-Ga for the $[G\text{-}Ga]_0$ of 25 and 50 g/L, respectively, which were similar to those using glucose, galactose and lactose alone at those saccharides concentration (Table 4.2).



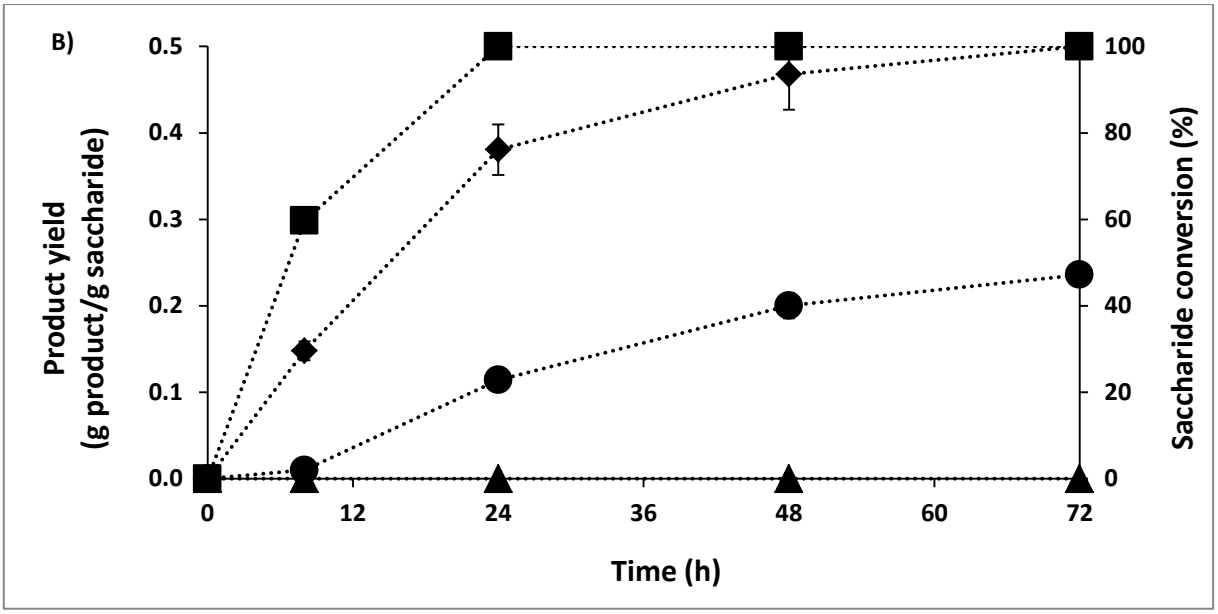


Figure 4.5: Glucose and galactose conversion and 2,3-BD and A yields as a function of time in the presence of *E. coli* JFR12. Two mixture $[G\text{-}Ga]_0$ were tested: 25 g/L (Figure 4.5A) and 50 g/L (Figure 4.5B). Figure 4.5A: Glucose conversion (□), galactose conversion (◊), 2,3-BD (○) and A (Δ). Figure 4.5B: Glucose conversion (■), galactose conversion (♦), 2,3-BD (●) and A (▲). Results are means ± SD of 2 experiments performed in duplicate.

On one hand, glucose was preferred over galactose as a carbon source by *E. coli* JFR12 for both $[G\text{-}Ga]_0$ as shown in Figure 4.5 (A and B). As reported by Luo et al. (2014), glucose is at the top of the saccharide hierarchy to be metabolized by *E. coli* causing the phenomenon called glucose carbon catabolite repression setting galactose use aside, which may explain the higher glucose conversion for $[G\text{-}Ga]_0$ at 25 and 50 g/L. On the other hand, the 2,3-BD yields obtained for $[G\text{-}Ga]_0$ at 25 and 50 g/L were similar to those obtained with galactose (Figure 4.3B); thus, this might confirm that galactose hampers the formation of 2,3-BD by acting on the enzymes involved in its production. Acetoin and Et yields were similar in presence of glucose, galactose, lactose and mixture G-Ga at 25 and 50 g/L, which suggests that their formation depends on the saccharide concentration and not on the kind of carbon source.

Figure 4.6 (A and B) presents the 2,3-BD yield obtained for the highest saccharide conversion at 25 and 50 g/L of saccharides (glucose, galactose, lactose and G-Ga). Figure 4.6A shows the 2,3-BD yield when saccharide conversion was 100%: glucose (48 h), galactose (48 h), lactose (48 h) and the mixture G-Ga (24 h) at 25 g/L. The 2,3-BD yields for glucose and lactose were around 0.35 g/g saccharide, whereas for galactose and the mixture G-Ga, it was 46% lower (0.19 g/g saccharide) ($p < 0.05$). Figure 4.6B shows that the 2,3-BD yields for glucose and lactose at a concentration of 50 g/L at 72 h were around 0.28 g/g saccharide and it was slightly smaller for galactose and the mixture G-Ga of around 0.25 g /g saccharide.

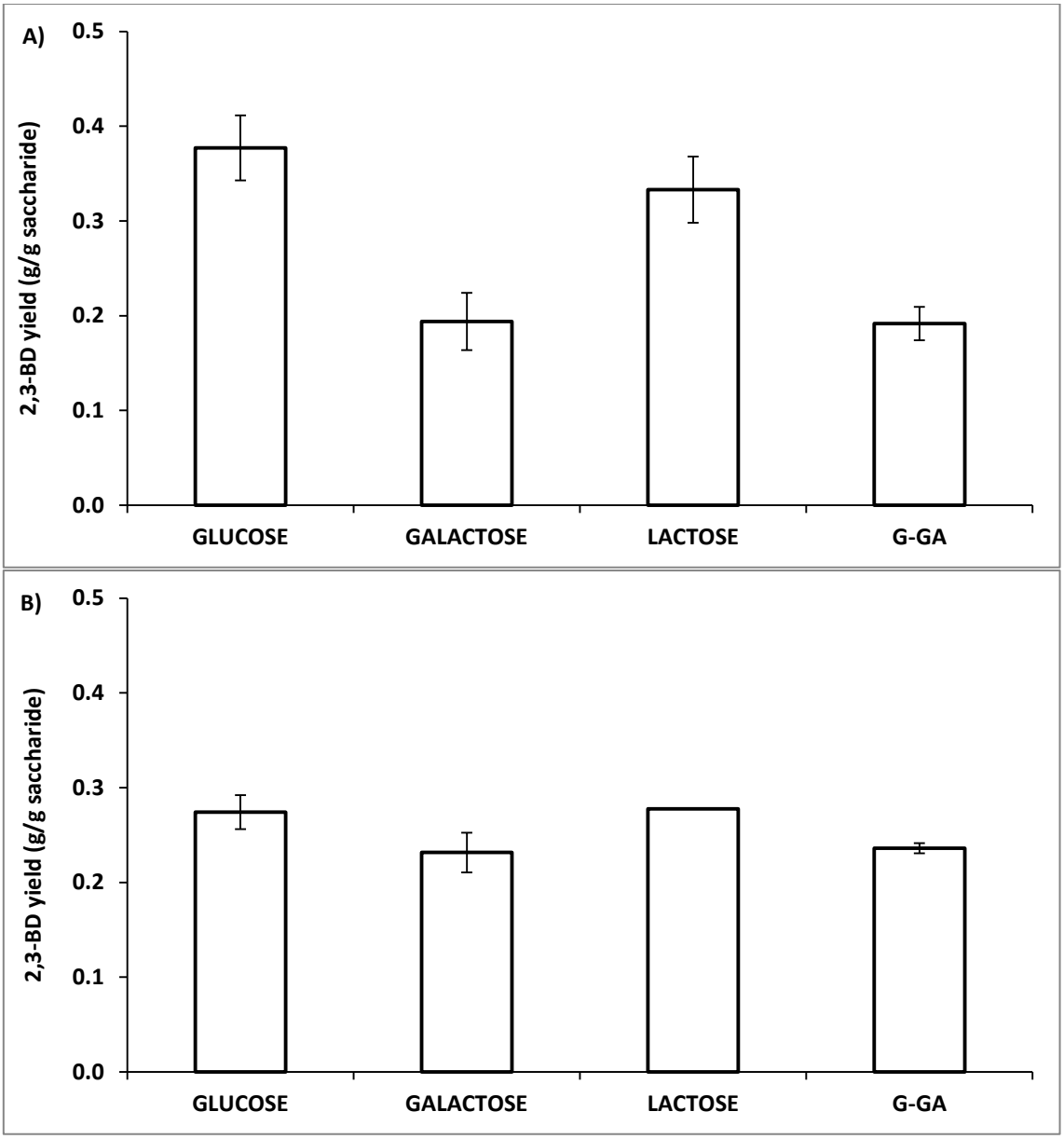


Figure 4.6: 2,3-BD yield at maximum saccharide conversion at 25 g/L (Figure 4.6A) and 50 g/L (Figure 4.6B) of saccharide concentration with *E. coli* JFR12. Figure 4.6A: 2,3-BD yield using glucose (48 h), galactose (48 h), lactose (48 h) and mixture G-Ga (24 h) when conversion was 100% in all cases. Figure 4.6B: 2,3-BD yield using glucose (conversion 94%), galactose (conversion 90%), lactose (conversion 96%) and mixture G-Ga at 72 h (conversion 100% for both monosaccharides). Results are means ± SD of 2 experiments performed in duplicate.

On one hand, the maximum conversion of saccharides (between 90 and 100% at 72 h) was similar at 50 g/L (Figures 4.2A, 4.3A, 4.4A and 4.5B). The explanation might be linked to the high saccharide concentration (50 g/L), which might cause the metabolism inhibition of *E. coli* JFR12. All bacteria have a substrate concentration limit where the bacterial metabolism may be inhibited if it is exceeded, affecting the maximum 2,3-BD yield (Chan et al. 2016, Priya et

al. 2016). This phenomenon was also observed in the present study comparing the 2,3-BD yields at 50 g saccharides/L (glucose, galactose, lactose and mixture G-Ga) since the maximum 2,3-BD was similar. This might confirm that the substrate inhibitory effect on the bacterial metabolism is caused by a high saccharide concentration whose range might be between 25 and 50 g/L for *E. coli* JFR12, explaining similar 2,3-BD yield (around 0.25 g/g saccharide) at 50 g/L obtained whatever the saccharide used. Lactose can be transformed into 2,3-BD as efficiently as glucose since galactose obtained from lactose hydrolysis does not impede the formation of 2,3-BD, which was clearly observed at 25 g/L of saccharides.

4.5.1.5 Whey

The genetically modified *E. coli* JFR12 was also tested to ferment lactose contained in W1 and PW1. In order to determine the effect of substrate concentration on 2,3-BD yield, W1 and PW1 were fermented using three $[Lac]_0$. The lactose concentration was adjusted by diluting W1 and PW1 with M9 medium. For W1, the $[Lac]_0$ tested were 15.5, 23 and 31 g/L while for PW1 17, 25.5 and 34 g/L were used. The fermentations were performed under the operating conditions shown in Table 1.

Figure 4.7 (A, B and C) shows the lactose conversion, 2,3-BD and A yields using whey as a function of time and $[Lac]_0$. For a $[Lac]_0$ of 15.5 g/L, the lactose conversion was 52% at 8 h and reached 100% at 24 h. On the other hand, using a $[Lac]_0$ of 23 g/L, the conversion increased with the time and reached 100% at 48 h; whereas with a $[Lac]_0$ of 31 g/L, the lactose conversion increased and reached 100% at 72 h (Figure 4.7A).

The 2,3-BD yield increased from 0.08 to 0.24 g/g lactose at 8 and 24 h, respectively in the presence of 15.5 g/L of lactose, and then decreased by 38% (0.15 g/g lactose) at 72 h ($p < 0.05$). Fermenting 23 g/L of lactose, the 2,3-BD yield increased from 0.04 g/g lactose at 8 h to 0.40 g/g lactose at 48 h remaining constant up to 72 h ($p < 0.05$). On the other hand, the 2,3-BD yield increased from 0.04 g/g lactose at 8 h to reach a maximum 2,3-BD yield of 0.43 g/g lactose at 72 h ($p < 0.05$) with 31 g/L of lactose (Figure 4.7B). Figure 4.7C shows that A yield increased from 0.03 g/g lactose at 24 h to 0.12 g/g lactose at 72 h for a $[Lac]_0$ of 15.5 g/L. Acetoin was not detected up to 72 h with 23 and 31 g/L of lactose, being the A yields 0.07 and 0.04 g/g lactose, respectively. These results were close to those obtained using lactose alone at 12.5 and 25 g/L in M9 (Figure 4.4B and C).

The formation of A was observed when the lactose conversion was 100% in presence of *E. coli* JFR12, i.e. at 24 h for the $[Lac]_0$ of 15.5 g/L and at 72 h for $[Lac]_0$ of 23 and 31 g/L. As mentioned before, the BDH enzyme catalyzes reversibly the reaction A $\rightleftharpoons$ 2,3-BD ($NAD^+/NADH + H^+$). The reaction is directed to A when lactose conversion is 100% and the pool of free NAD^+ increases, which decreases the amount of 2,3-BD. This phenomena was clearly observed in the present study for the lowest $[Lac]_0$ of 15.5 g/L.

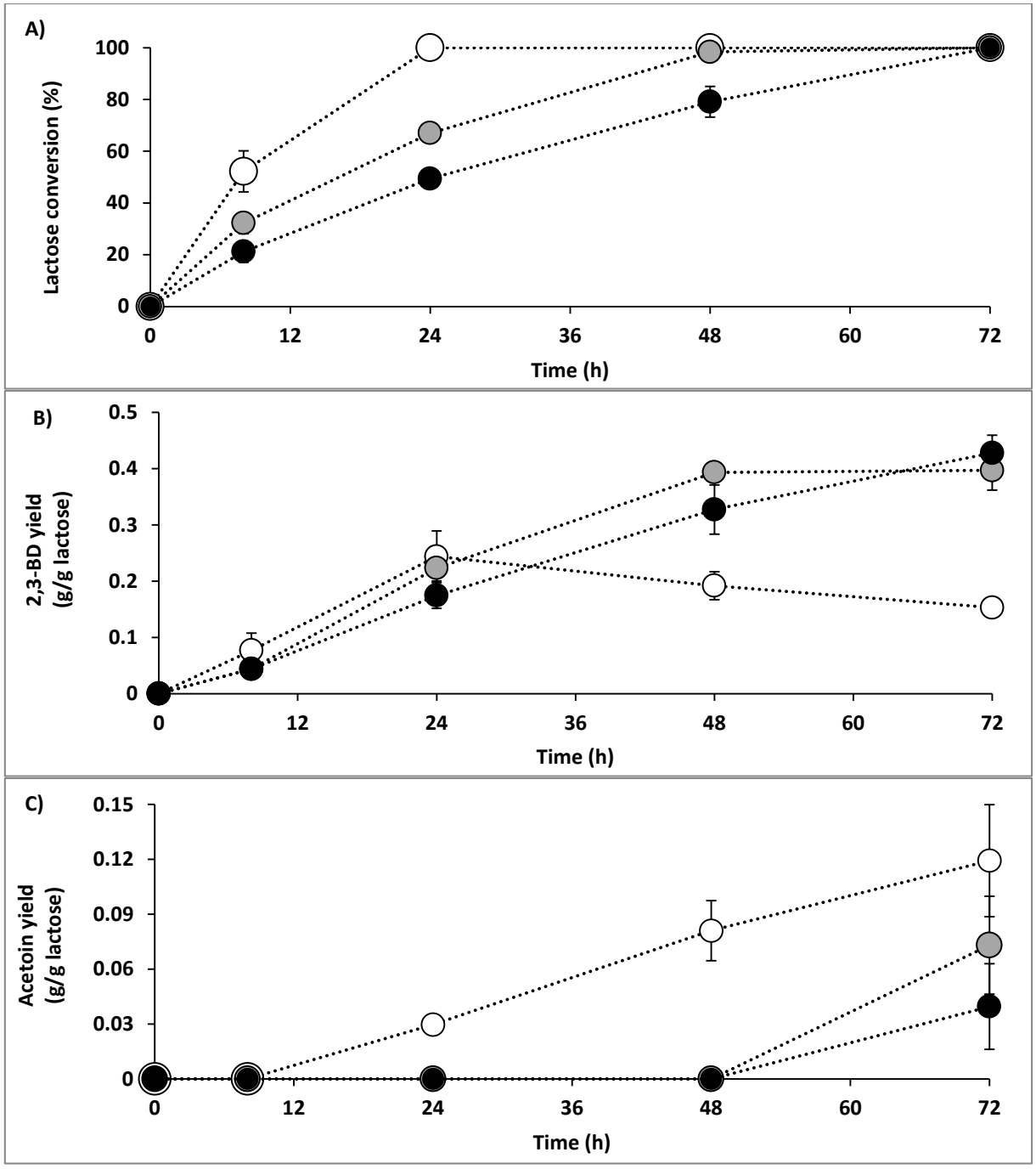


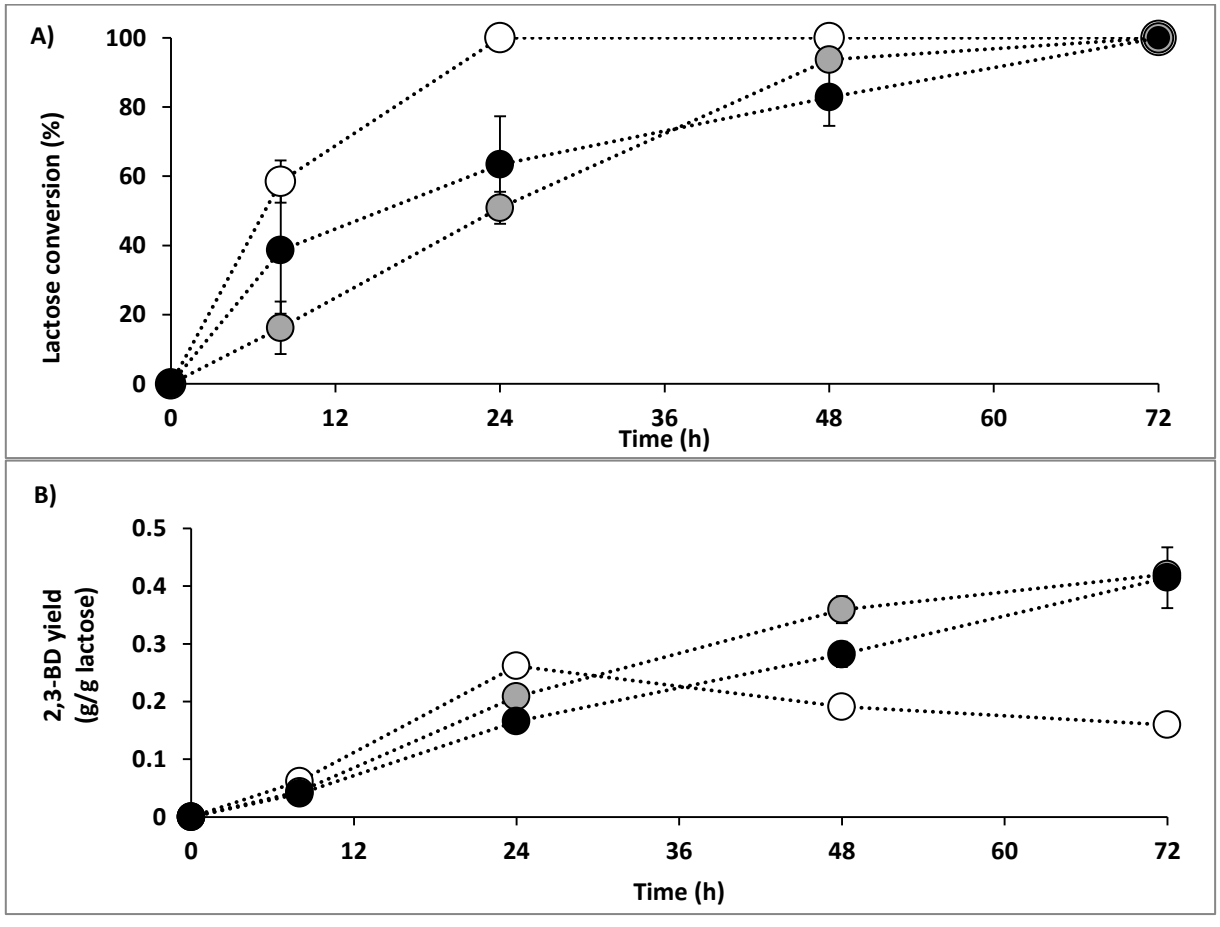
Figure 4.7: Lactose conversion (Figure 4.7A), 2,3-BD yield (Figure 4.7B) and A yield (Figure 4.7C) fermenting W1 diluted with M9 as a function of time in the presence of *E. coli* JFR12. Lactose concentration: 15.5 (○), 23 (●) and 31 (●) g/L of lactose. Results are means ± SD of 2 experiments performed in duplicate.

4.5.1.6 Permeate whey

Figure 4.8 (A, B and C) presents the lactose conversion, 2,3-BD and A yields as a function of time using PW1 diluted with M9. Fermenting 17 g/L of lactose the conversion was 59% at 8 h and then reached 100% at 24 h. In the presence the $[Lac]_0$ of 25.5 g/L, the lactose conversion increased from 54% at 8 h to 100% at 72 h. Similarly, the lactose conversion increased from 39% at 8 h to 100% at 72 h with 34 g/L (Figure 4.8A).

Similarly to W1, the 2,3-BD yield increased from 0.06 g/g lactose at 8 h to 0.26 g/g lactose at 24 h for the lowest $[Lac]_0$ of 17 g/L. Then, the 2,3-BD yield decreased by 38% down to 0.16 g/g lactose at 72 h ($p < 0.05$). For the 2 other $[Lac]_0$, the 2,3-BD yield increased from 0.04 g/g lactose at 8 h to achieve the maximum 2,3-BD yield of 0.42 g/g lactose at 72 h ($p < 0.05$), as shown Figure 4.8B.

Acetoin was detected at 24 h fermenting 17 g/L of lactose, being its yield 0.03 g/g lactose increasing up to 0.12 g/g lactose at 72 h. On the other hand, A reached a yield of 0.07 g/g lactose for 25.5 g/L of lactose, whereas it was not detected at 34 g/L at 72 h (Figure 4.8C).



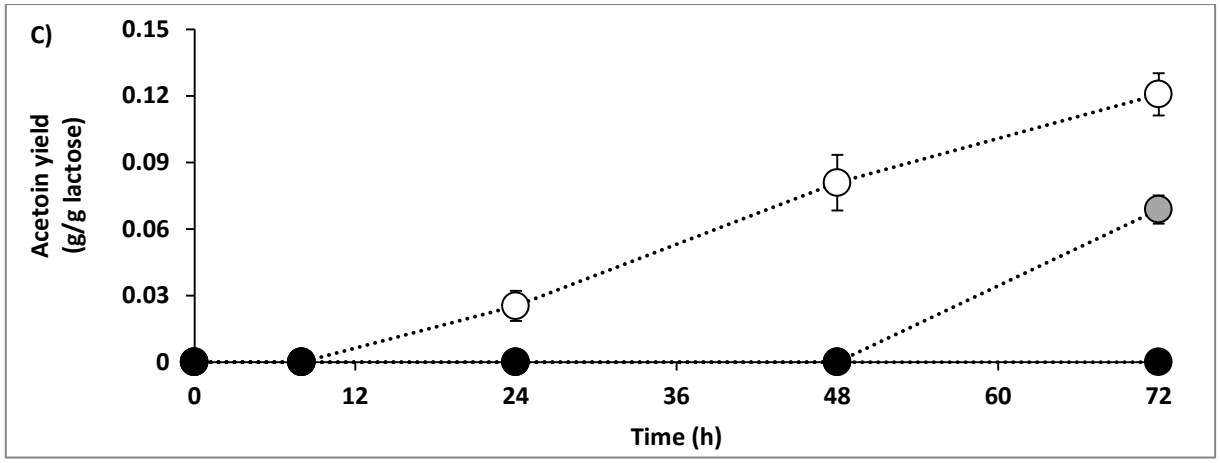


Figure 4.8: Lactose conversion (Figure 4.8A), 2,3-BD yield (Figure 4.8B) and A yield (Figure 4.8C) fermenting PW1 diluted with M9 as a function of time in the presence of *E. coli* JFR12. Lactose concentration: 17 (○), 25.5 (●) and 34 (●) g/L. Results are means ± SD of 2 experiments performed in duplicate.

As observed in Figures 4.7 and 4.8, the yields of 2,3-BD and A were quite similar for W1 and PW1 at the 3 dilution ratios, showing that the 2,3-BD and A yields are mainly determined by the lactose concentration. For W1 and PW1, the highest 2,3-BD yields were obtained at the dilution ratios of 75:25 and 100:0 (v/v). Therefore, the best results were obtained in absence of M9 because minerals and nutrients contained in W1 and PW1 (Panesar et al. 2007, USDEC 2017) could support the growth of *E. coli* JFR12 and the formation of 2,3-BD without adding other macro or micronutrients present in the synthetic culture medium. For this reason, neither W1 nor PW1 were diluted with M9 for the subsequent experiments. In addition, the use of PW1 will be preferred because it is an end effluent in dairy industry which should be valorized.

To our best knowledge, the fermentation of W and PW has not been performed using *E. coli* strains. These effluents have been fermented with other bacteria, e.g. *E. aerogenes*, *K. oxytoca*, *K. pneumoniae* or *Lactococcus lactis* to produce 2,3-BD obtaining yields in the range from 0.20 to 0.43 g/g lactose (Kandasamy et al. 2016, Lee and Maddox 1986, Perego et al. 2000, Vishwakarma 2014). For example, Perego et al. (2000) fermented W at a $[Lac]_0$ of 20 g/L using *E. aerogenes* NCIMB 10102 obtaining a 2,3-BD yield of 0.43 g/g lactose with an inoculum of 0.25 g/L and at 39ºC, pH 6 and 150 rpm for 250 h. Whereas, Vishwakarma (2014) reported a 2,3-BD yield of 0.20 g/g lactose (2,3-BD yield calculated from the data provided by the author) in the presence of *K. oxytoca* NRRl-13-199 using PW (30-32.5 g/L of lactose) and 1% (v/v) of inoculum at 30°C, pH 6.5 and 60 rpm for 96 h.

The use of *E. coli* JFR12 in the present study has several advantages in comparison to the previous mentioned bacteria: i) the strain *E. coli* JFR12 is harmless for human, whereas *E. aerogenes*, *K. oxytoca* and *K. pneumoniae* are known pathogens (iGEM 2017, PHAC 2017); ii) the highest 2,3-BD yield (0.43 g/g lactose) obtained with *E. coli* JFR12 is similar to the best yields obtained with pathogen strains; and iii) the fermentation time of 72 h to obtain the highest 2,3-BD yield in the present study was shorter than the times required for fermentations using *E. aerogenes*, *K. oxytoca* and *K. pneumoniae*, which vary from 96 to 250 h.

4.5.2 Effect of fermentation parameters

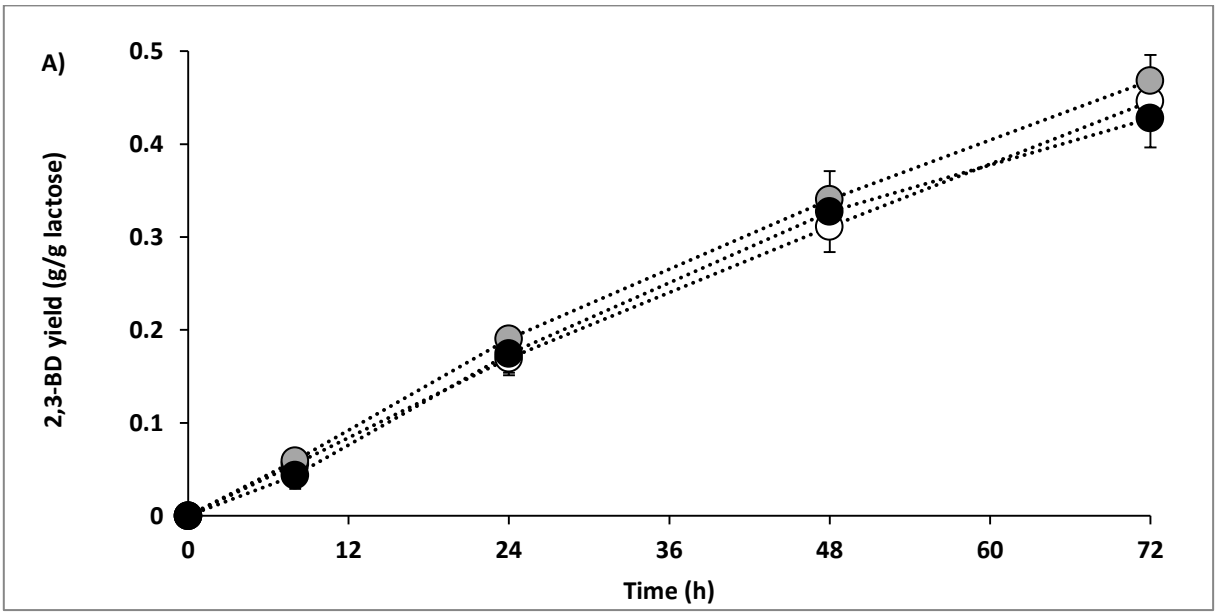
The effect of 3 operating conditions (initial pH, inoculum size and agitation rate) on 2,3-BD yield was studied. Both W and PW were fermented during 72 h using *E. coli* JFR12 under the operating conditions mentioned before (Table 4.1).

4.5.2.1 Initial pH

Figure 4.9 (A and B) present the evolution of 2,3-BD yield as a function of time and initial pH (pH_0 = 6.5, 7.0 and 7.4) for W1 and PW1 fermentation, respectively. Lactose conversions were 100% after 72 h of fermentation whatever the pH_0 (data not shown).

Between 8 and 72 h, the 2,3-BD yield varied from 0.05 to 0.47 g/g lactose for W1 and from 0.04 to 0.44 g/g lactose using PW1 for the 3 initial pH_0s tested. Therefore, for the range of pH_0 tested, the 2,3-BD yield did not present any difference for W1 or PW1 ($p < 0.05$) in presence of *E. coli* JFR12.

The pH was uncontrolled during W1 and PW1 fermentation; for this reason, the pH decreased to values around 5.8 for all pH_0. The acidification of the fermentation medium could be caused by the organic acids formation like PA. The pH stayed at 5.8 because metabolic pathways adjusted to avoid a higher medium acidification, favoring the formation of 2,3-BD. This mechanism to control the internal pool of PA was suggested by Mazumdar et al. (2013).



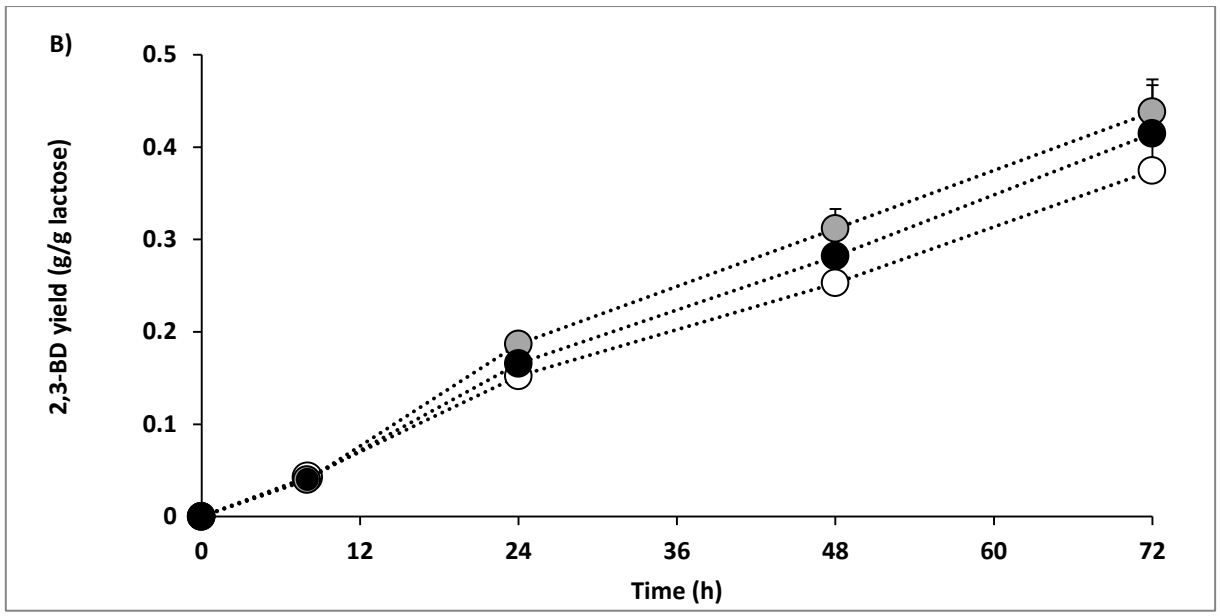


Figure 4.9: 2,3-BD yield fermenting W1 (Figure 4.9A) and PW1 (Figure 4.9B) as a function of time in the presence of *E. coli* JFR12 at different initial pH: 6.5 (○), 7.0 (●) and 7.4 (●). Results are means ± SD of 2 experiments performed in duplicate.

4.5.2.2 Inoculum size

Figures 4.10 and 4.11 show the effect of the inoculum size (5, 7.5 and 10%, v/v) on the lactose conversion and the 2,3-BD yield as a function of time for the fermentation of W1 and PW1.

The lactose conversion with a 5% (v/v) of inoculum was close to 78%, whereas the lactose conversion was 100% for 7.5 and 10% (v/v) of inoculum after 72 h (Figure 4.10A). The 2,3-BD yield increased from 8 to 72 h for W1 and PW1. At 72 h, the highest 2,3-BD yield was 0.38 g/g lactose for 5% (v/v) of inoculum and 0.45 g/g lactose for 7.5 and 10% (v/v) of inoculum size fermenting W1 (Figure 4.10B). Lactose conversions were similar for PW1 and W1, being 79, 89 and 100% for 5, 7.5 and 10% (v/v), respectively at 72 h (Figure 4.11A). The maximum 2,3-BD yield was 0.33 g/g lactose for 5% (v/v) of inoculum and near 0.40 g/g lactose in the presence of 7.5 and 0.46 g/g lactose for 10% (v/v) of inoculum at 72 h (Figure 4.11B).

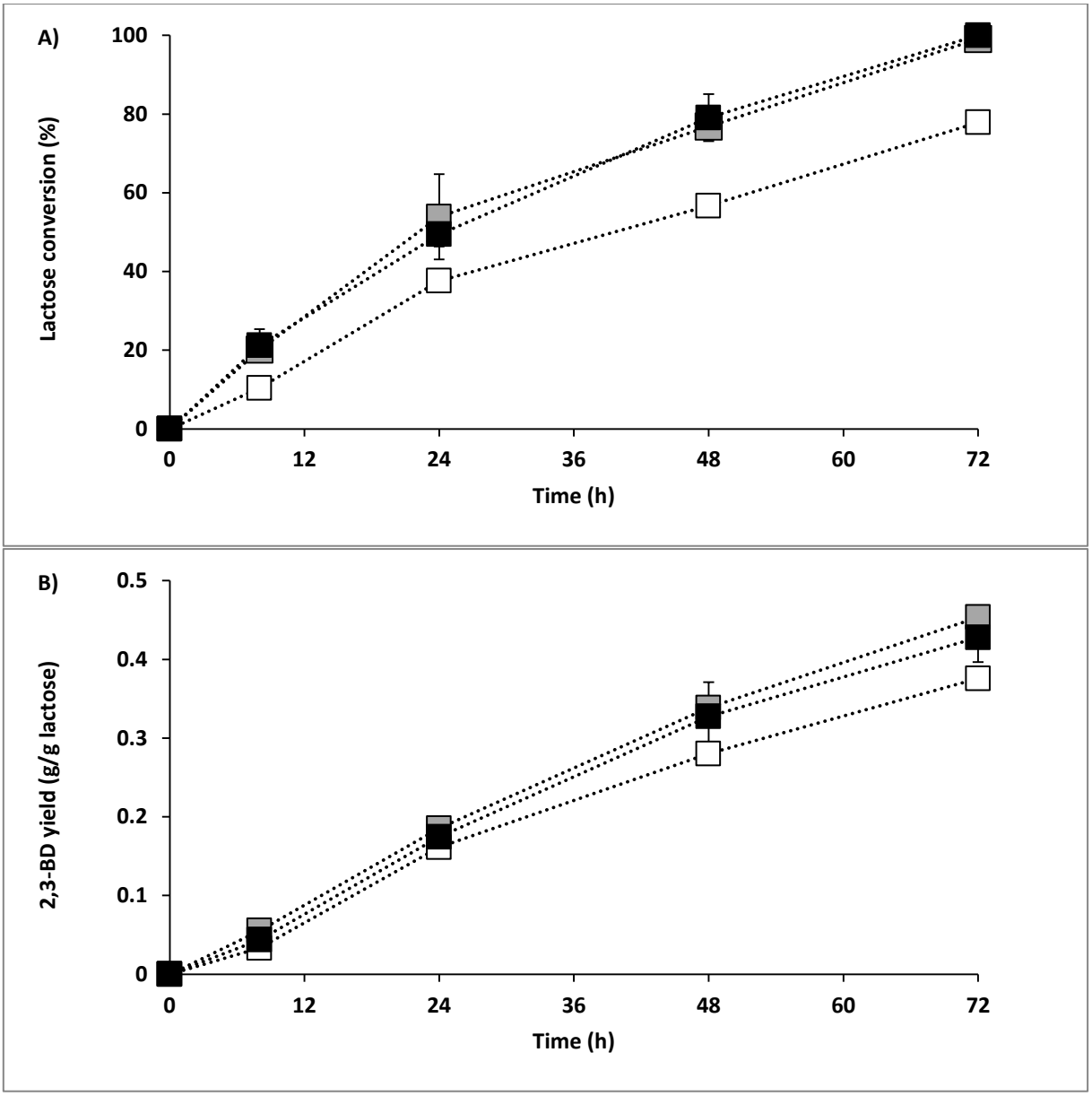


Figure 4.10: Lactose conversion (Figure 4.10A) and 2,3-BD yield (Figure 4.10B) fermenting W1 as a function of time in the presence of *E. coli* JFR12 at different inoculum sizes (%, v/v). Inoculum size: 5% (□), 7.5% (■) and 10% (■). Results are means ± SD of 2 experiments performed in duplicate.

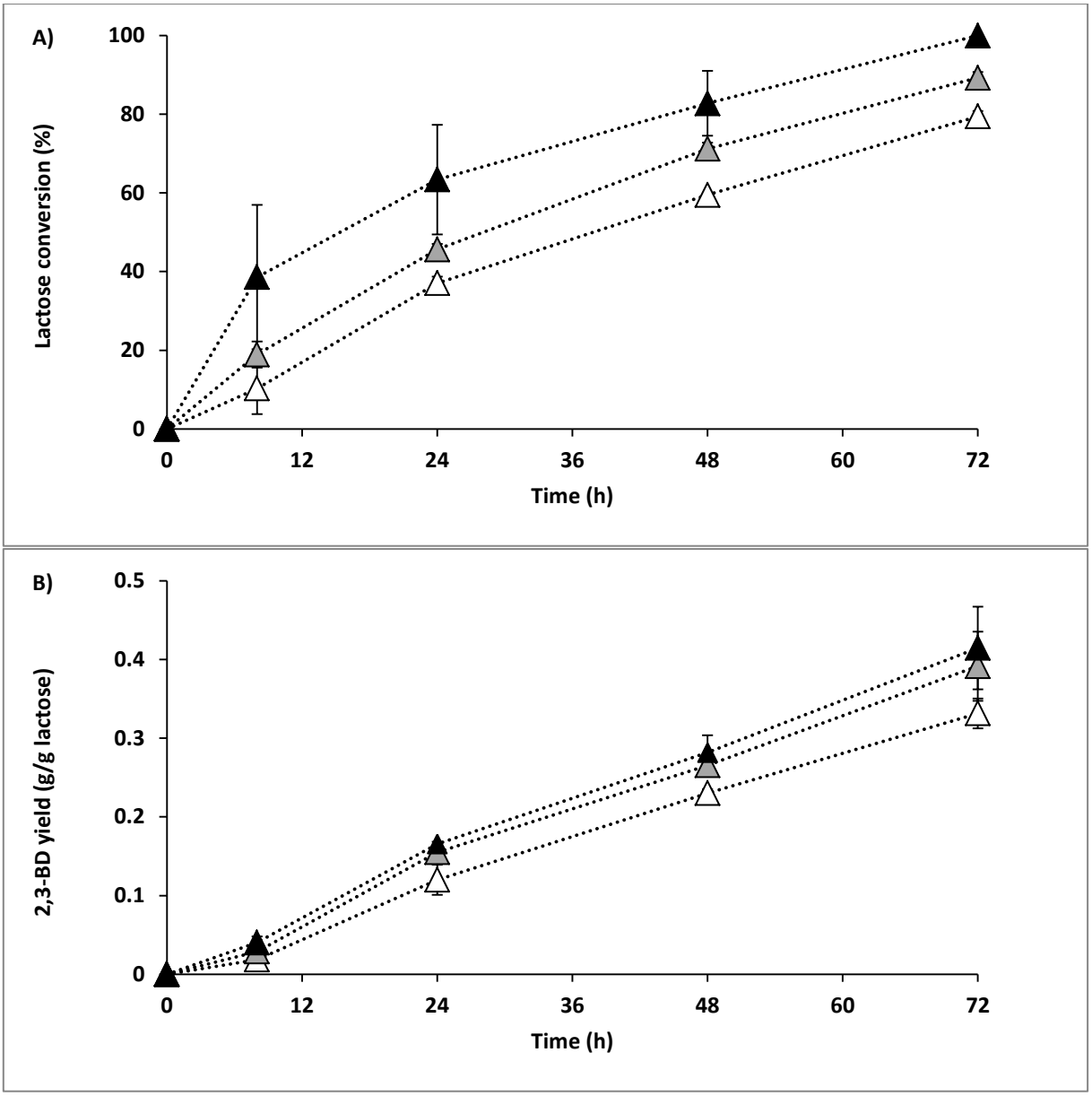


Figure 4.11: Lactose conversion (Figure 4.11A) and 2,3-BD yield (Figure 4.11B) fermenting PW1 as a function of time in the presence of E. coli JFR12 at different inoculum sizes (%, v/v). Inoculum size: 5% (Δ), 7.5% (▲) and 10% (▲). Results are means ± SD of 2 experiments performed in duplicate.

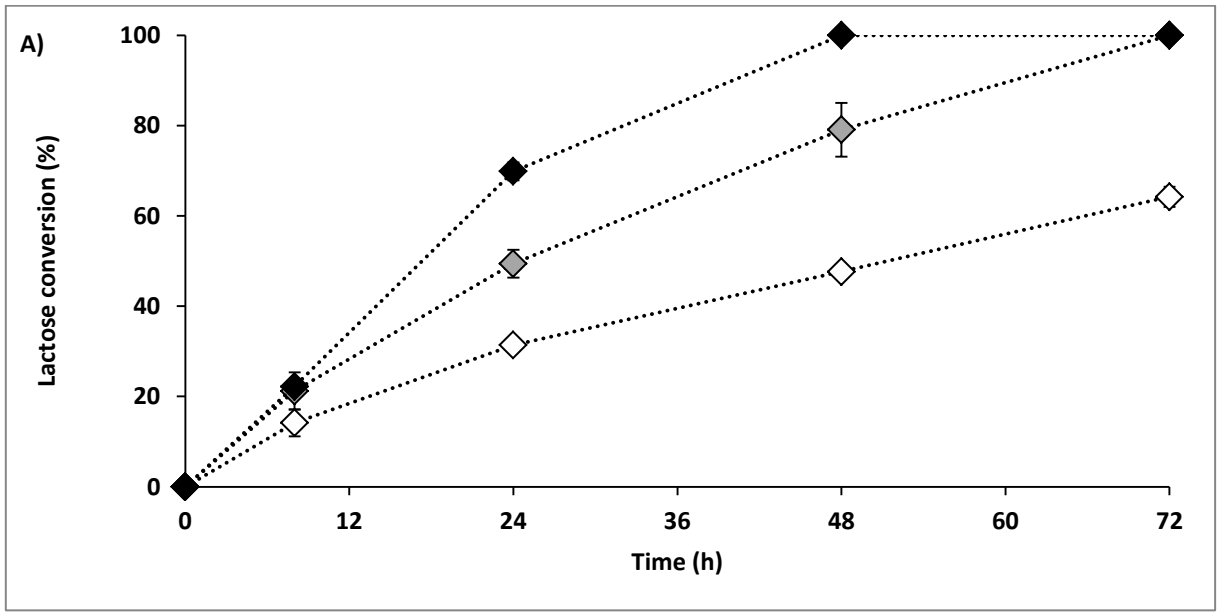
The 2,3-BD yields (0.38 and 0.33 g/g lactose using W1 and PW1, respectively) obtained at 5% (v/v) of inoculum were around 20% lower ($p < 0.05$) compared to the other 2 inoculum size tested. This could be due to there was lactose in the culture medium without consuming at 72 h. In this way, the use of 5% (v/v) of inoculum might be a critical cellular density to reach a high 2,3-BD yield for a fermentation time of 72 h. Therefore, the use of a small inoculum size (5%, v/v) decreased the 2,3-BD yield, which in accordance with other studies (Okonkwo et al. 2017). For example, Perego et al. (2003) tested an inoculum of *B. licheniformis* of 0.5 and 10 g/L of dry weight (number of viable bacteria not defined) at a concentration of 20 g/L of

glucose, 37ºC, pH 6 and 150 rpm (fermentation time not defined); the 2,3-BD yield increased with inoculum size from 0.1 to 0.35 g/g glucose.

4.5.2.3 Agitation

Figures 4.12 and 4.13 (A and B) show the effect of agitation rate on lactose conversion and 2,3-BD yield as a function of time for W1 and PW1, respectively. Table 3 shows the 2,3-BD, A and ABD (A + 2,3-BD) yield at 48 and 72 h for W1 and PW1 as a function of agitation rate.

In the case of W1, at 50 rpm, lactose conversion increased with time from 14% at 8 h to 64% at 72 h, whereas for 100 and 200 rpm, it was close to 20% at 8 h and 100% at 72 h and 48 h, respectively (Figure 4.12A). The 2,3-BD yield increased with time, up to 0.26 g/g lactose at 72 h and 50 rpm. Using an agitation rate of 100 rpm, the 2,3-BD yield reached 0.43 g/g lactose at 72 h; whereas at 200 rpm, the maximum 2,3-BD yield (0.23 g/g lactose) was reached at 24 h and then decreased down to 0.14 g/g lactose ($p < 0.05$) at 72 h (Figure 4.12B). Acetoin was not detected at 50 rpm during the fermentation and A yield was 0.04 g/g lactose at 72 h and 100 rpm. However, A yield increased up to 0.23 g/g lactose ($p < 0.05$) at 72 h and 200 rpm (Table 4.3).



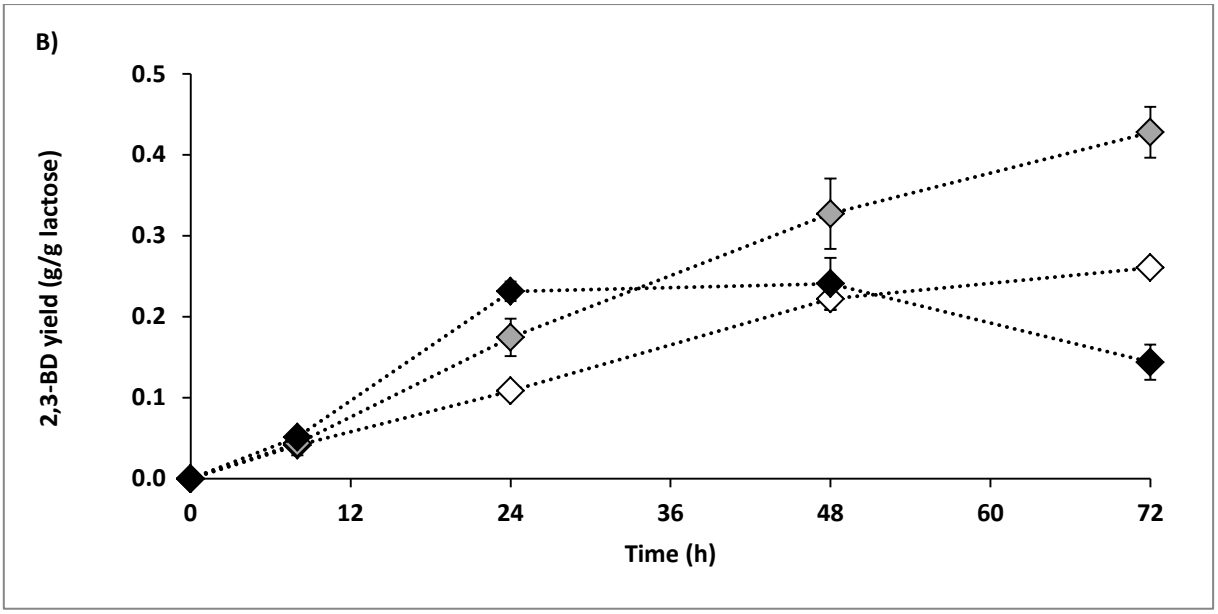


Figure 4.12: Lactose conversion (Figure 4.12A) and 2,3-BD yield (Figure 4.12B) fermenting W1 as a function of time in the presence of *E. coli* JFR12 at different agitation rates. Agitation rate: 50 (◊), 100 (♦) and 200 rpm (♦). Results are means ± SD of 2 experiments performed in duplicate.

As shown in Table 4.3, at 200 rpm, the reversible reaction (2,3-BD → A) was clearly observed at 48 and 72 h, when lactose conversion was 100% using W1. In this case, the ABD yield was practically constant (variation of 13%) showing that 2,3-BD might be used by *E. coli* JFR12 as a carbon source.

When PW1 was fermented, the lactose conversion varied with the time quite similarly to what has been observed for W1 at 50 rpm; 14% at 8 h of fermentation and reaching a maximum of 60% at 72 h. At 100 rpm, lactose conversion increased from 39% at 8 h up to 100% after 72 h. However, at 200 rpm, lactose conversion was 20% at 8 h and the maximum lactose conversion was 90% at 72 h (Figure 4.13A). At 50 rpm, 2,3-BD was not detected for the first 8 h of fermentation, increasing its yield up to 0.26 g/g lactose at 72 h. The highest 2,3-BD yield was reached at 100 rpm for 72 h, achieving 0.42 g/g lactose. However, at 200 rpm, the maximum 2,3-BD yield was 0.26 g/g lactose at 72 h (Figure 4.13B). On the other hand, A was not detected either at 50 or 100 rpm during fermentation. However, A yield at 48 and 72 h was 0.05 g/g lactose at 200 rpm even whether lactose was present in the medium (Table 4.3).

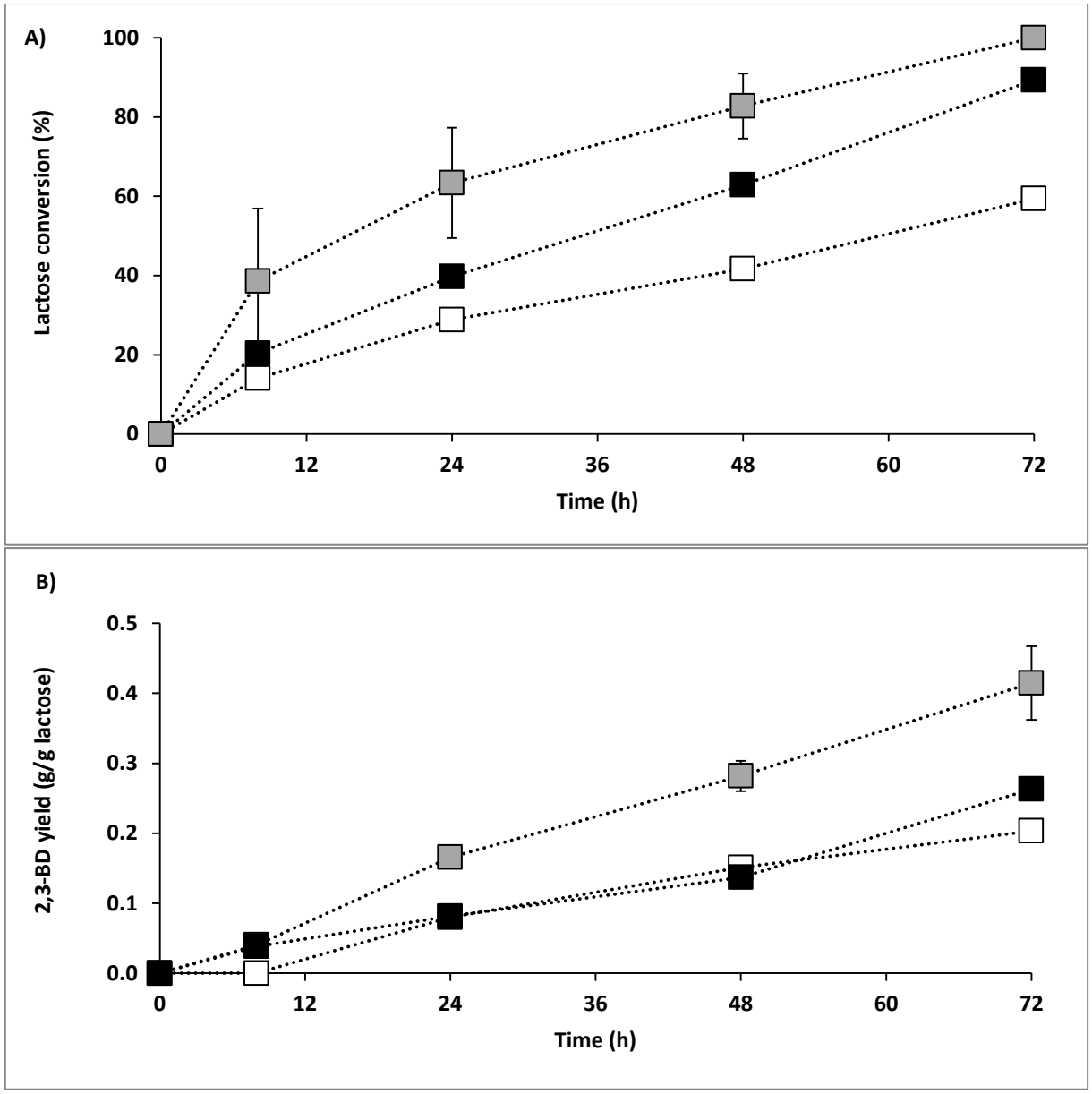


Figure 4.13: Lactose conversion (Figure 4.13A) and 2,3-BD yield (Figure 4.13B) fermenting PW1 as a function of time in the presence of *E. coli* JFR12 at different agitation rates. Agitation rate: 50 (□), 100 (■) and 200 rpm (■). Results are means ± SD of 2 experiments performed in duplicate.

Table 4.3: Acetoin, 2,3-BD and ABD yields as a function of time fermenting W1 and PW1 at 37ºC, 1 atm and initial pH 7.4 for 48 and 72 h in flasks varying the agitation rate.

W1				
Agitation rate (rpm)	Time (h)	2,3-BD yield (g/g lactose)	A yield (g/g lactose)	ABD yield (g/g lactose)
50	48	0.22	0.00	0.22
	72	0.26	0.00	0.26
100	48	0.33	0.00	0.33
	72	0.43	0.04	0.47
200	48	0.24	0.19	0.43
	72	0.14	0.23	0.37
PW1				
Agitation rate (rpm)	Time (h)	2,3-BD yield (g/g lactose)	A yield (g/g lactose)	ABD yield (g/g lactose)
50	48	0.15	0.00	0.15
	72	0.20	0.00	0.20
100	48	0.28	0.00	0.28
	72	0.42	0.00	0.42
200	48	0.14	0.05	0.19
	72	0.26	0.05	0.31

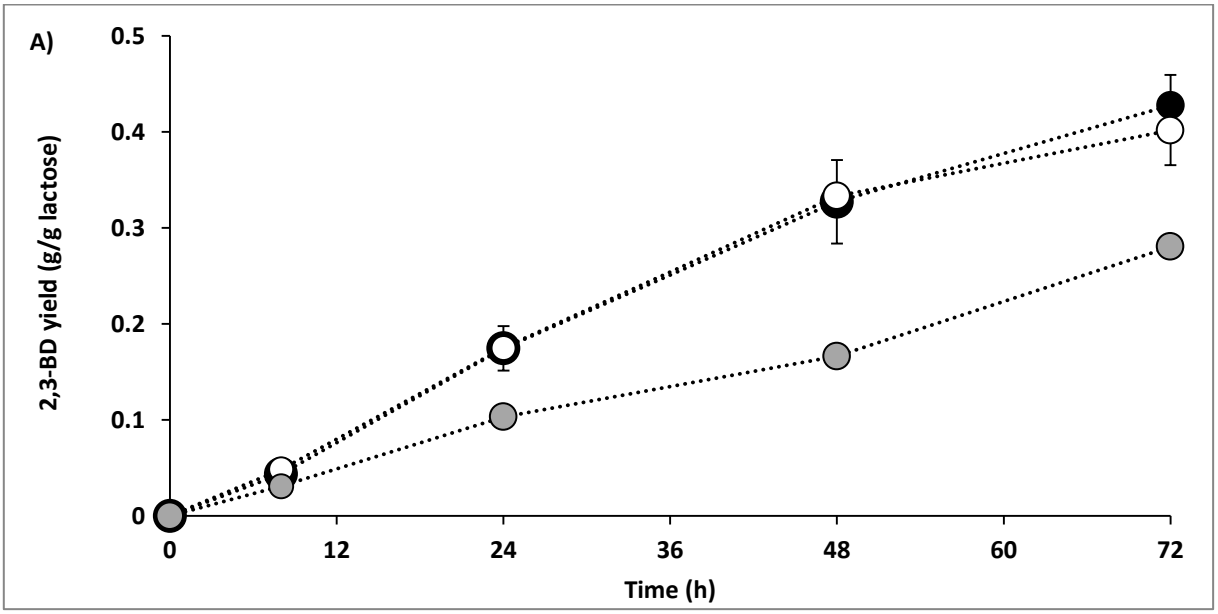
Comparing the fermentation of W1 and PW1, the conversion of lactose was higher for W1 compared to PW1 at 200 rpm. This can be due to the presence of more nutrients in W1, because the PW1 is the by-product issued from protein removal of W1 (Smith et al. 2016). This causes that PW1 has less nitrogen and amino acids than W1. The ABD yield was slightly higher for W1, confirming the possible effect of nutrients on ALDC enzyme as suggested by Cho et al. (2015). Figures 4.12B and 4.13B show that the agitation rate affected lactose conversions and 2,3-BD yields. On one hand, at a low agitation rate (50 rpm), the lactose conversion was lower for both W1 and PW1. At a relatively high agitation rate (200 rpm) the formation of 2,3-BD and A are linked together and the maximum 2,3-BD yield is lower compared to the one obtained at 100 rpm using W1 and PW1. A high agitation would provide higher oxygen transfer to the culture medium and could cause the inactivation of enzyme ALDC (Shi et al. 2014). In this way, a secondary reaction spontaneously may occur transforming α-acetolactate into diacetyl, which is transformed into A generating free NAD^+. When A is transformed into 2,3-BD, free NAD^+ is also obtained and thus the amount of free NAD^+ increases in the broth (Figure 4.1). In this way, when lactose conversion was 100%, the reversible reaction A $\rightleftharpoons$ 2,3-BD was activated, decreasing the amount of 2,3-BD in the broth and increasing the A yield, as observed for 200 rpm using W1. In addition, according to Priya et al. (2016), A can accumulate as agitation rate increases because the oxygen dissolved in the broth and the oxidation potential of system also increases, causing the rise of NAD^+ in the broth even if the carbon source conversion is not 100%. This is in accordance with the assay of PW1 fermentation at 200 rpm of the present study.

4.5.3 Fermentations in bioreactor

4.5.3.1 Anaerobic fermentation

Whey and PW fermentations were also performed in a 2 L bioreactor with a working volume of 0.8 L. The operating conditions are shown in Table 4.1. Air was not supplied and thus anaerobic conditions were rapidly achieved, decreasing the dissolved oxygen (DO) near 30% (w/v) in the first 10 minutes and no DO was detected (DO close to 0%) in the following 10 minutes. For these experiments, W2 and PW2 were used. Fermentations of W2 and PW2 with or without dilution in distilled water were also performed in flasks; W1 and PW1 were used as controls.

Figure 4.14 (A and B) shows the 2,3-BD yield as a function of time for W1 and W2 fermentation in flasks (Figure 4.14A) and in bioreactor (Figure 4.14B). The 2,3-BD yields obtained in flasks using W1 ($[Lac]_0$ of 31 g/L) and diluted W2 ($[Lac]_0$ of 31 g/L) increased with time and were quite similar (nearly 0.40 g/g lactose) at 72 h. In contrast, the 2,3-BD yield obtained at 72 h using W2 ($[Lac]_0$ of 51 g/L) was around 30% lower than the one reached with W1 and diluted W2 ($[Lac]_0$ 31 g/L) ($p < 0.05$). For fermentation in bioreactor, at 72 h, 2,3 BD yields of 0.26 and 0.23 g/g lactose were obtained using W1 and diluted W2 ($[Lac]_0$ 31 g/L), respectively. These 2,3 BD yields were 41% lower than those observed in flasks. On the other hand, the 2,3-BD yield using W2 ($[Lac]_0$ of 51 g/L) in bioreactor was 0.18 g/g lactose (22% lower than the 2,3-BD yield obtained with 31 g/L of lactose). Acetoin was not detected either in flasks or bioreactor using W2 at $[Lac]_0$ of 31 and 51 g/L (Figure 4.14B).



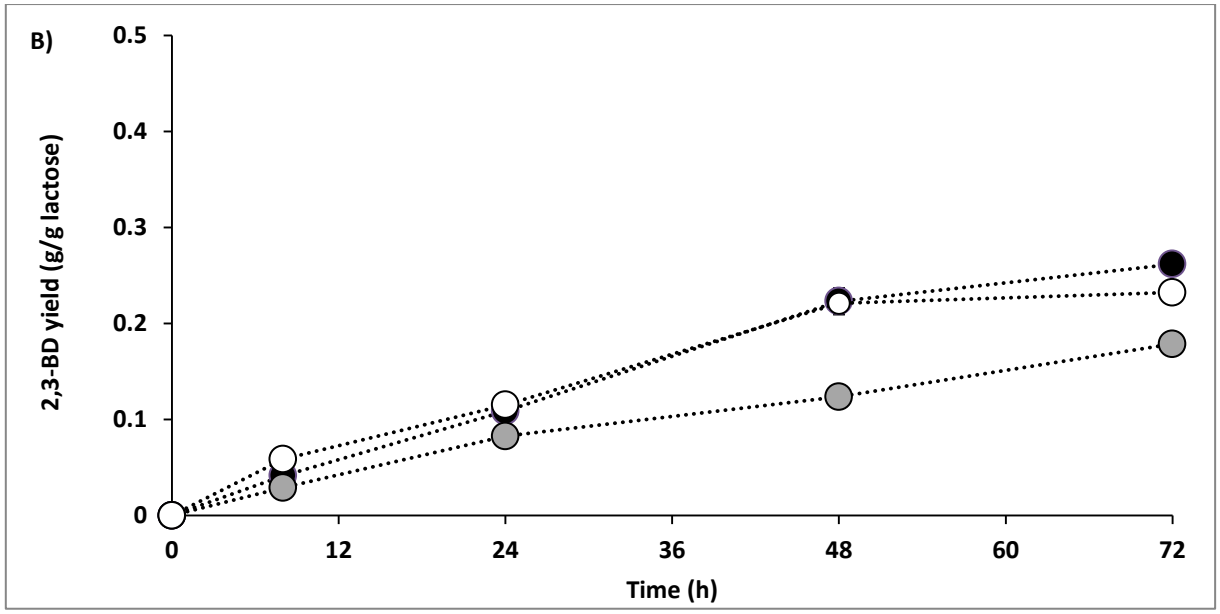
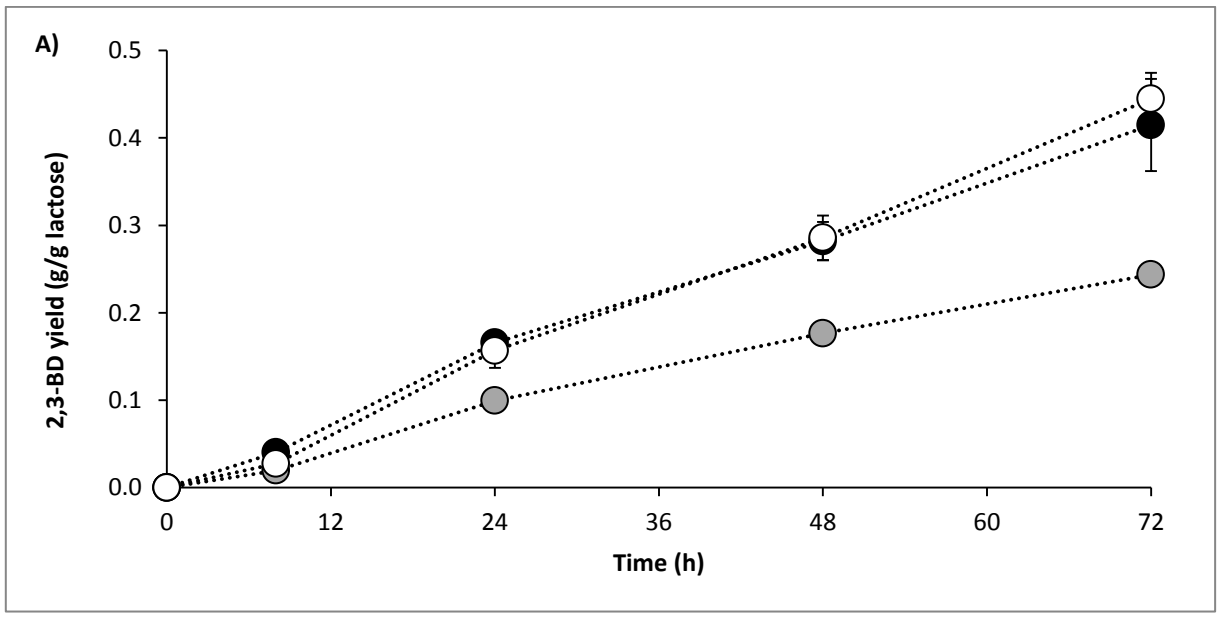


Figure 4.14: 2,3-BD yield as a function of time fermenting W1 and W2 in the presence of *E. coli* JFR12 in flask (Figure 4.14A) and bioreactor (Figure 4.14B). Figure 4.14A: W1 (●), W2 undiluted (●) and W2 diluted (○). Figure 4.14B: W1 (●), W2 undiluted (●) and W2 diluted (○). Results are means ± SD of an experiment performed in duplicate.

Figure 4.15 (A and B) shows the 2,3-BD yield as a function of time for PW1 and PW2 fermentation in flasks (Figure 4.15A) and in bioreactor (Figure 4.15B). The 2,3-BD yield increased with the fermentation time and was similar at 72 h using PW1 ($[Lac]_0$ of 34 g/L) and diluted PW2 ($[Lac]_0$ of 34 g/L) with around 0.42 g/g lactose, while it was decreased by nearly the half ($p < 0.05$) with PW2 ($[Lac]_0$ of 47 g/L). The 2,3-BD yield using diluted PW2 ($[Lac]_0$ of 34 g/L) in bioreactor was also smaller (close to 60%) compared to fermentations in flasks ($p < 0.05$). Acetoin was not detected for any fermentation.



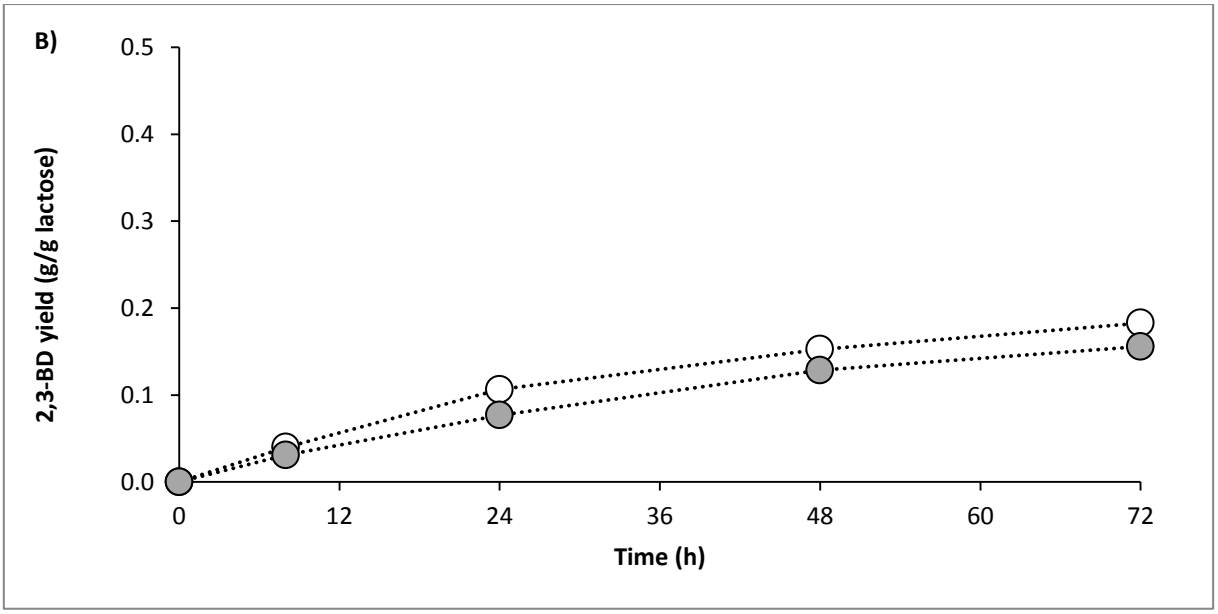


Figure 4.15: 2,3-BD yields as a function of time fermenting PW1 and PW2 in the presence of *E. coli* JFR12 in flask (Figure 4.15A) and bioreactor (Figure 4.15B). Figure 4.15A: PW1 (●), PW2 undiluted (●) and PW2 diluted (○). Figure 4.15B: PW2 undiluted (●) and PW2 diluted (○). Results are means ± SD of an experiment performed in duplicate.

Results showed that the 2,3-BD yields obtained in flasks were similar using W1 and diluted W2 ($[Lac]_0$ of 31 g/L). In addition, the 2,3-BD yields were higher using low lactose concentrations (W1 and diluted W2) than using higher lactose concentrations (W2). Similar results were obtained with PW. On the other hand, the 2,3-BD yield obtained using W2 and PW2 ($[Lac]_0$ of around 50 g/L) in flasks fermentation was similar to the one obtained with lactose (50 g/L) in M9 in flasks (Figure 4B). This confirms that the 2,3-BD accumulation in the broth is mainly a function of $[Lac]_0$ regardless of culture medium (M9, W or PW). In addition, these results can confirm that the threshold of lactose concentration may be around 35 g/L of lactose since the 2,3-BD yield increased compared to the results obtained at 25 g/L of lactose and decreased using around 50 g/L of lactose.

The amount of oxygen (O_2) in the broth is linked to the bacterial growth and thus to the formation of 2,3-BD (Ji et al. 2009a). However, microaerobic conditions may be used to improve the 2,3-BD yield (Mazumdar et al. 2013). Under microaerobic conditions, the amount of O_2 (less than 6.7 mg O_2/L at 37ºC) is limited leading to a suitable bacterial population and avoiding the inhibition of ALS and BDH enzymes, increasing the 2,3-BD formation (Missouri 2017, Yang et al. 2011). In this way, the assays performed in the 2 L bioreactor (strict anaerobic conditions) with W2 and PW2 led to lower 2,3-BD yields (up to 43% in the case of W2 and 59% for PW2) compared to those carried out in flasks, where the agitation could permit to dissolve some atmospheric O_2.

4.5.3.2 Aerated bioreactor

The effect of aeration to ferment diluted W2 ($[Lac]_0$ of 31 g/L) in the 2 L bioreactor was tested. The air flow rate was 2 L/min in a working volume of 0.8 L, equivalent to 2.5 vvm of aeration rate. The DO slightly decreased from the beginning of the fermentation of diluted W2 to 1% (w/v) after 12 h of fermentation. Figure 4.16 shows the lactose conversion, 2,3-BD, A and ABD yields as a function of time.

The lactose conversion reached 100% after 24 h of fermentation. The 2,3-BD yield increased from 0.08 g/g lactose at 8 h to 0.40 g/g lactose at 24 h and decreased to reach around 0.26 g/g lactose at 72 h. Acetoin was not detected within 24 h, while its yield reached 0.21 g/g lactose at 48 h and remained nearly constant until 72 h. The ABD yield was practically constant (around 0.40 g/g lactose) from 24 to 72 h. In fact, when the main carbon source (i.e. lactose) is not present in the culture medium (lactose conversion of 100%), the pool of NAD^+ increases, causing the use of 2,3-BD as a carbon source, decreasing its yield (observed after 24 h) and transforming 2,3-BD into A.

The maximum 2,3-BD yield (0.40 g/g lactose) was slightly lower at 24 h to that obtained in flask (0.43 g/g lactose) at 72 h under the same operating conditions. The air supply favored the lactose conversion (100%) and the 2,3-BD formation, reducing the time to reach the maximum 2,3-BD yield compared to the fermentations performed in flasks. However, 2,3-BD was transformed into A after 24 h. After 24 h, the ABD yield was nearly constant up to 72 h, which points out that 2,3-BD was used as a carbon source by means of BDH enzyme. Hence, on one hand, in an aerated bioreactor, it is possible to obtain the maximum 2,3-BD yield within 24 h; whereas on the other hand, after 24 h, the formation of A occurs in the presence of *E. coli* JFR12, being possible maximizing the production of either 2,3-BD or A.

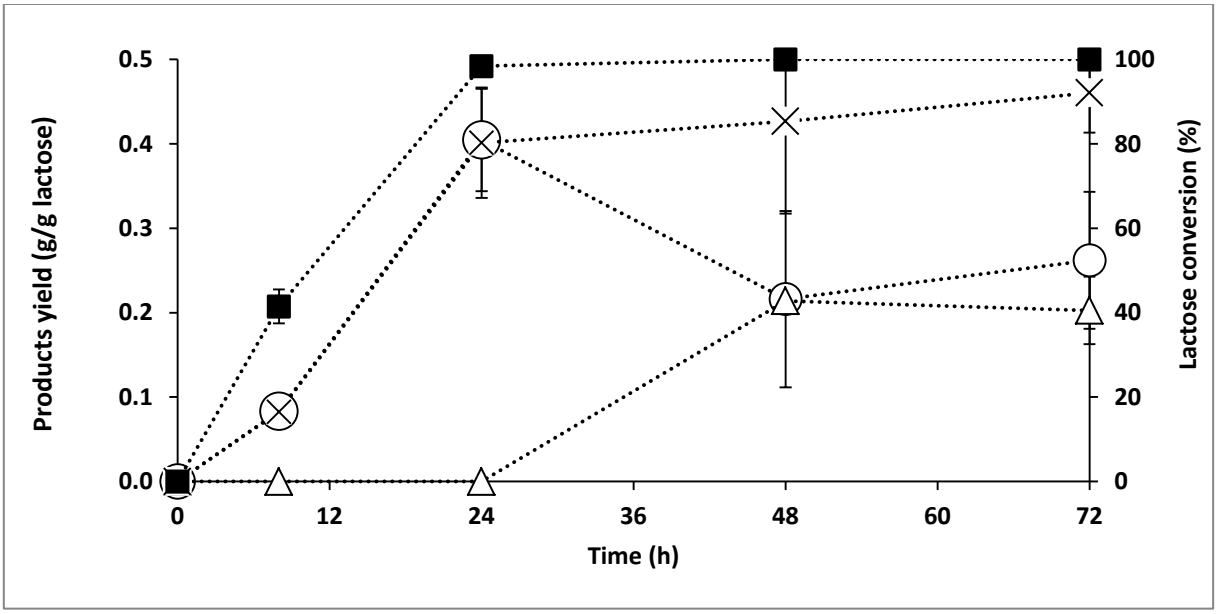


Figure 4.16: Lactose conversion (■), 2,3-BD (○), A (Δ) and ABD (×) yields fermenting diluted W2, $[Lac]_0$ 31 g/L, as a function of time in the presence of *E. coli* JFR12 in a bioreactor of 2 L. Results are means ± SD of an experiment performed in duplicate.

The 2,3-BD yield at 24 h in the aerated bioreactor was more than 2 fold higher than that in flask under the same operating conditions. According to Shi et al. (2014) and Yang et al. (2011), a suitable amount of O_2 may improve the activity of ALS and BDH enzymes. Therefore, the results obtained in the present study show that the use of 2 L/min was appropriate to avoid the inactivation of ALS and BDH enzyme.

4.6. Conclusion

Fermentation of different saccharides derived from whey to produce 2,3-butanediol and acetoin using a genetically modified strain of *Escherichia coli* K12 MG1655 (*E. coli* JFR12) was performed. Different concentrations (12.5, 25 and 50 g/L) of glucose, galactose, lactose and a mixture of glucose-galactose were tested in a supplemented M9 culture medium at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm in 0.5 L flask (0.2 L of working volume). Similar 2,3-BD yields (around 0.38 g/g saccharide) for 25 g/L of glucose or lactose were obtained at 48 h. Galactose gave lower 2,3-BD yields (50% less at 25 g/L and 48 h). However, galactose released by lactose hydrolysis seems not to interfere with the formation of 2,3-BD.

Mixing whey (W1, 31 g/L of lactose) or permeate whey (PW1, 34 g/L of lactose) with and without M9 (50:50, 75:25 and 100:0, v/v) showed that highest 2,3-BD yields were obtained in absence of M9 in the presence of *E. coli* JFR12, reaching 2,3-BD yields of 0.43 and 0.42 g/g lactose using W1 and PW1, respectively.

The influence of the initial pH (6.5, 7.0 and 7.4), inoculum size (5.0, 7.5 and 10.0%, v/v) and agitation rate (50, 100 and 200 rpm) were tested at 37ºC and 1 atm in flask of 0.5 L (0.2 L of working volume). The initial pH did not have a significant influence, being the 2,3-BD yields 0.47 and 0.44 g/g lactose for W1 and PW1, respectively. On the other hand, the use of 5% (v/v) of inoculum size gave a 2,3-BD yield slightly lower (14% and 18% using W1 and PW1, respectively) compared to 7.5 and 10% (v/v). The highest agitation rate (200 rpm) favored the acetoin formation; whereas the 2,3-BD yield was not improved. The 2,3-BD yield was 23% and 67% lower at 200 rpm compared to the one obtained at 100 rpm at 48 and 72 h, respectively using W1. On the other hand, fermenting PW1 at 200 rpm, the 2,3-BD yield was 61% and 38% lower at 48 h and 72 h, respectively, compared to the results at 100 rpm.

Another batch of W (W2, 51 g/L of lactose) and PW (PW2, 47 g/L of lactose) were tested in flasks. Higher lactose concentrations conducted to lower 2,3-BD yields: 30% and 46% using W2 and PW2, respectively.

Whey 2 and PW2 fermentation were also performed in a 2 L bioreactor under anaerobic conditions, which reduced the 2,3-BD formation for all the fermentation times, whereas the supply of air (2.5 vvm) reduced the time by 3 fold to obtain the highest 2,3-BD yield of 0.40 g/g lactose at 24 h, in comparison to the fermentations performed in flasks.

This study demonstrated that lactose from whey and permeate whey can be fermented in the presence of *E. coli* JFR12 in order to obtain 2,3-BD or acetoin according the experimental conditions.

4.7 Acknowledgement

Michèle Heitz is grateful to Fond Québécois de la Recherche sur la Nature et les Technologies (FRQNT) in partnership with MAPAQ-NOVALAIT (Innovation en Production et en Transformation Laitières) for the grant attributed to the research program "Développement d'une biotechnologie verte innovatrice pour valoriser des résidus fromagers". The authors are grateful to the laboratory technicians (Department of chemical and biotechnological engineering of Université de Sherbrooke) Isabelle Arsenault and Valérie Larouche for their support and help. The authors would like to particularly thank Pr. Sébastien Rodrigue and the master student Jean-François Rousseau (Department of biology of Université de Sherbrooke) for providing the genetically modified strain *E. coli* JFR12. The authors would like to thank Pr. Denis Groleau (Department of chemical and biotechnological engineering of Université de Sherbrooke) for lending his bioreactor to perform the fermentations shown in the present chapter. Michèle Heitz would also like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for a financially contribution at the end of the study.

CHAPTER 5. Conclusion

The valorization of byproducts of the agro-food industry like polysaccharides via enzymatic catalysis is currently a great challenge, which has to overcome in the following years. Diverse bacterial strains, such as *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae* or *Serratia marcescens*, allow to transform polysaccharides into 2,3-butanediol (2,3-BD), a platform molecule. These microorganisms are divided into two group: i) natural producers of 2,3-BD like *E. cloacae* and *K. pneumoniae*, and ii) genetically modified strains like *E. coli*.

The biovalorization of saccharides via fermentation allows unlike the chemical process (160 and 220ºC, and 50 bar) working at softer operating conditions (temperatures between 25 and 40ºC, atmospheric pressure and pH close to 7.0) to produce of 2,3-BD without using petroleum sources. Each bacterial strain is differently affected by the operating conditions like kind and concentration of nutrients in the culture medium, temperature, pH, agitation, aeration, etc. In the present study, the main objective has been to study the ability of a genetically modified strain of *Escherichia coli* (*E. coli* JFR12) K12 MG1655 to transform lactose contained in whey (W) and permeate whey (PW) into 2,3-BD.

The first specific objective was to measure and quantify the amount of bacteria in the inoculum before performing the fermentation. In addition, a comparison between the wild (ECW) and the genetically modified (*E. coli* JFR1) strains of *E. coli* was carried out using the colony-forming unit (CFU) method. The *E. coli* JFR1 strain presented a CFU value of $6.5x10^8$ CFU/mL, which was 41% lower compared to the value obtained in the presence of ECW. Therefore, the genetic modifications performed in ECW to obtain ECGM seem to affect the bacterial growth of *E. coli*.

The second specific objective was to evaluate the effect of the glucose concentration on the ABD (acetoin + 2,3-BD) production using 4 culture media (LB, M9, M63 and MOPS) in the presence of *E. coli* JFR1. The glucose concentrations in the 4 culture media were 4, 12.5 and 25 g/L. The highest ABD yield was close to 0.25 g ABD/g glucose at 48 and 72 h using 12.5 and 25 g/L of glucose, respectively, in LB, M9 and M63 at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 6.5 and 100 rpm. On the other hand, the use of MOPS culture medium decreased the ABD yields for those glucose concentrations (0.18 and 0.14 g ABD/g glucose, respectively, at 48 h). The selected culture medium was M9 since it presented an optimized relationship between the ABD yield and culture medium price.

The third specific objective was to study the effect of urea ($(NH_2)_2CO$) and sodium nitrate ($NaNO_3$) as an additional nitrogen source (ANS) and their concentration in M9 culture medium on ABD yield in the presence of *E. coli* JFR1. The use of urea as an ANS improved the ABD yield up to 0.27 g ABD/g glucose at 96 h in the presence of 15 g/L of urea fermenting 25 g/L of glucose at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 6.5 and 100 rpm. The ABD formation was hampered when the ANS was $NaNO_3$. Therefore, the ABD formation depends on the chemical nature of the nitrogen source.

The fourth specific objective of the study was to test the effect of glucose, galactose and lactose concentration in M9 culture medium to produce 2,3-BD in the presence of another

mutant strain of *E. coli*: *E. coli* JFR12. This strain was obtained to improve the 2,3-BD production. The fermentation of glucose and lactose (25 g/L) provided similar 2,3-BD yields (close to 0.38 g 2,3-BD/g saccharide) at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 6.5 and 100 rpm for 48 and 72 h, respectively. Nevertheless, with galactose, lower 2,3-BD yields (0.19 g 2,3-BD/g galactose) at 48 h compared to those obtained with glucose and lactose. In order to understand these results, a mixture of glucose and galactose (12.5 and 25 g/L of each monosaccharide) were fermented. In the presence of galactose as a sole carbon source, the 2,3-BD concentration was lower compared to that one obtained when galactose was generated from lactose hydrolysis.

The fifth specific aim was to evaluate the 2,3-BD yield using mixtures of W (31 g/L of lactose, W1) and PW (34 g/L of lactose, PW1) in the presence of *E. coli* JFR12. In this case, W1 and PW1 were mixed with M9; three dilutions (W1 or PW1/W9) (50:50, 75:25 and 100:0, v/v) were tested. The maximum 2,3-BD yields (0.43 and 0.42 g 2,3-BD/g lactose with W1 and PW1, respectively) were reached at 72 h in absence of M9 culture medium (100:0, v/v) at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm. Hence, *E. coli* JFR12 was able to transform efficiently the lactose contained in both effluents to 2,3-BD, with a value near the maximum 2,3-BD theoretical yield (0.52 g 2,3-BD/g lactose).

The sixth specific goal was focused on the influence of three parameters (initial pH, inoculum size and agitation rate) on 2,3-BD yield fermenting W1 and PW1 in absence of M9 culture medium and in the presence of *E. coli* JFR12. The inoculum size of 7.5% (v/v) gave a similar 2,3-BD yield to that one obtained using 10% (v/v) of inoculum, close to 0.45 and 0.42 g 2,3-BD/g lactose for W1 and PW1, respectively at 37ºC, 1 atm, initial pH 7.4 and 100 rpm. Concerning the effect of the initial pH, no influence on the 2,3-BD yield was observed. In this particular case, in the presence of W1 and PW1, the 2,3-BD yields were 0.47 and 0.44 g 2,3-BD/g lactose, respectively at 10% (v/v) of inoculum, 37ºC, 1 atm and 100 rpm. As far as the W1 fermentation at 10% (v/v) of inoculum, 37ºC, 1 atm and initial pH 7.4 is concerned, an agitation rate of 200 rpm gave a maximum 2,3-BD yield of 0.23 g/g lactose at 24 h. This yield was two folds lower than the maximum yield obtained at 100 rpm at 72 h. For the fermentation of PW1 under similar operating conditions, an agitation rate of 200 rpm provided the same effects on the 2,3-BD yield.

A new batch of W (W2) and PW (PW2) were fermented in 0.5 L flasks and in a 2 L bioreactor (without aeration) at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm. The W2 and PW2 contained, respectively, a lactose concentration of 51 and 47 g/L. Both effluents were directly fermented and diluted with distilled water to obtain a lactose concentration of 31 and 34 g/L of lactose, respectively, in 0.5 L flasks and the 2 L bioreactor. In flasks, the 2,3-BD yields obtained with diluted W2 and PW2 were comparable to those obtained with W1 and PW1. In both cases (W1 and W2 or PW1 and PW2) under anaerobic conditions, the 2,3-BD yields were lower.

Afterwards, in a 2 L bioreactor, the effect of the air at 2 L/min on 2,3-BD formation was tested in the presence of diluted W2. The addition of air increased the 2,3-BD yield by 135% (0.40 g 2,3-BD/g lactose) compared to the yield obtained in flask (0.5 L). By comparison, the 2,3-BD yields obtained in flasks for 24 h at 10%, v/v, of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100

rpm were lower. Hence, the use of a bioreactor adding air allowed rapidly obtaining a high yield (reduction of the fermentation time from 72 h to 24 h).

Therefore, the main objective of this study, the optimization of W and PW fermentation by a mutant strain of *E. coli*, was achieved. The fermentation of both effluents gave high 2,3-BD yields, up to 89 and 84% of the theoretical yield (W and PW, respectively). In this way, the strain *E. coli* JFR12 can be considered a good candidate to valorize W and PW in order to produce 2,3-BD.

On the other hand, it has been demonstrated that both whey and permeate whey are two appropriate culture media for bacteria like *E. coli* JFR12. In this way, both industrial effluents can become innocuous for the environment since they can be valorized by a green biotechnology. It would be good to consider an in-depth study of the operating parameters of fermentation in order to determine the optimal ones, above all the aeration rate in a fermentor as this study has shown. Afterwards, the study at fed-batch and continuous operating modes in a pilot scale would be strongly interesting in order to implement the fermentation of whey and permeate whey at an industrial scale.

5.1 Conclusion in French

La valorisation des sous-produits de l'industrie agro-alimentaire tels les polysaccharides par catalyse enzymatique reste un défi considérable pour les années à venir. Diverses souches bactériennes, telles qu'*Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae* ou *Serratia marcescens*, permettent de transformer les polysaccharides en 2,3-butanediol (2,3-BD), une molécule plateforme. Ces microorganismes se divisent en deux catégories: i) ceux produisant naturellement du 2,3-BD i.e. *E. cloacae* et *K. pneumoniae*, et ii) ceux génétiquement modifiés tels *Escherichia coli*.

La biovalorisation des saccharides par fermentation permet contrairement au procédé chimique (160 à 220ºC, 50 bar) de travailler sous des conditions opératoires douces (température variant entre 25 et 40ºC, pression atmosphérique et pH 7.0) et de produire du 2,3-BD non issu de ressources pétrolières. Chaque souche bactérienne est affectée différemment en fonction des conditions opératoires telles le type et la concentration de nutriments dans le milieu de culture, la température, le pH, la vitesse d'agitation, l'aération, etc.

Lors de cette étude, l'objectif principal a été d'étudier l'activité d'une souche génétiquement modifiée d'*E. coli* (*E. coli* JFR12) K12 MG1655 afin de transformer le lactose contenu dans le lactosérum (W) et le perméat de lactosérum (PW) en 2,3-BD.

Le premier objectif spécifique a été de mesurer et de quantifier la quantité de bactéries présentes dans l'inoculum avant de réaliser des fermentations. De plus, une comparaison entre les souches sauvage (ECW) et génétiquement modifiée (*E. coli* JFR1) d'*E. coli* a été accomplie en utilisant la méthode des unités formatrices de colonies (CFU). Pour la souche *E. coli* JFR1, la valeur de CFU est de 6.5×10^8 CFU/mL, et est inférieure de 41% à celle obtenue

en présence d'ECW. Par conséquent, les modifications génétiques réalisées sur ECW pour obtenir *E. coli* JFR1 semblent affecter la croissance d'*E. coli*.

Le deuxième objectif spécifique a été d'évaluer l'effet de la concentration de glucose sur la production d'ABD (Acétoïne + 2,3-BD) en utilisant 4 milieux de culture (LB, M9, M63 et MOPS) en présence d'*E. coli* JFR1. Les concentrations de glucose dans les 4 milieux de culture ont été de 4, 12.5 et 25 g/L. Le rendement le plus élevé en ABD a été environ 0.25 g ABD/g glucose à 48 et 72 h en utilisant 12.5 et 25 g/L de glucose respectivement dans LB, M9 et M63 à 10% (v/v) d'inoculum, 37ºC, 1 atm, pH initial 6.5 et 100 rpm. D'un autre côté, l'utilisation du milieu MOPS a diminué les rendements en ABD pour les mêmes concentrations de glucose (0.18 et 0.14 g ABD/g glucose, respectivement, à 48 h). Le milieu de culture qui a été finalement retenu est le M9 car il permet une optimisation des rendements en ABD et des coûts.

Le troisième objectif spécifique a été d'évaluer l'effet de l'urée ($(NH_2)_2CO$) et du nitrate de sodium ($NaNO_3$) en tant que source additionnelle d'azote (SAA) et de leurs concentrations dans le milieu de culture M9 sur le rendement en ABD en présence d'*E. coli* JFR1. L'utilisation d'urée comme SAA a amélioré le rendement en ABD jusqu'à 0.27 g ABD/g glucose en 96 h en présence de 15 g/L d'urée lors de la fermentation de 25 g/L de glucose à 10% (v/v) d'inoculum, 37ºC, 1 atm, pH initial 6.5 sous une agitation de 100 rpm. La formation d'ABD est affectée lorsque la SAA est du $NaNO_3$. Par conséquent, la formation d'ABD dépend de la nature chimique de la source d'azote.

Le quatrième objectif spécifique de cette étude a été d'étudier l'effet de la concentration de glucose, de galactose et de lactose dans le milieu de culture M9 pour produire du 2,3-BD en présence d'une autre souche d'*E. coli* i.e. *E. coli* JFR12. Cette souche devait permettre d'améliorer la production de 2,3-BD. La fermentation du glucose et du lactose (25 g/L) a conduit à des rendements similaires en 2,3-BD (proche de 0.38 g 2,3-BD/g saccharide) à 10% (v/v) d'inoculum, 37ºC, 1 atm, pH initial de 6.5 et 100 rpm à 48 et 72 h respectivement. Cependant avec le galactose, les rendements en 2,3-BD étaient plus faibles (0.19 g 2,3-BD/g galactose) à 48 h comparés à ceux obtenus avec le glucose et le lactose. Pour comprendre ces résultats, un mélange de glucose et de galactose (concentration de 12.5 et de 25 g/L de chaque monosaccharide) a été fermenté. En présence de galactose comme seule source de carbone, la concentration en 2,3-BD est moins élevée par rapport à celle obtenue lorsque le galactose est issu de l'hydrolyse du lactose.

Le cinquième objectif spécifique a été d'évaluer le rendement en 2,3-BD d'un mélange de W (31 g/L de lactose, W1) et de PW (34 g/L de lactose, PW1) en présence d'*E. coli* JFR12. W1 ou PW1 a été introduit dans le milieu de culture M9; trois dilutions (W1 ou PW1 / M9) (50:50, 75:25 et 100:0, v/v) ont été testées. Les rendements maxima en 2,3-BD (0.43 et 0.42 g 2,3-BD/g lactose avec W1 et PW1, respectivement) ont été obtenus après 72 h en absence du milieu de culture M9 avec 10% (v/v) d'inoculum, à 37ºC, sous 1 atm, avec un pH initial de 7.4 et une vitesse d'agitation de 100 rpm. Ainsi la souche d'*E. coli* JFR12 a permis de transformer efficacement le lactose contenu dans les deux effluents en produisant du 2,3-BD, avec une valeur proche du rendement théorique maximum (0.52 g 2,3-BD/g lactose).

Le sixième objectif spécifique a porté sur l'influence de trois paramètres (pH initial, volume d'inoculum et vitesse d'agitation) sur le rendement en 2,3-BD obtenu lors de la fermentation de W1 ou PW1 en absence du milieu de culture M9 et en présence d'*E. coli* JFR12. Le pourcentage d'inoculum à 7.5% (v/v) a donné un rendement similaire en 2,3-BD à celui obtenu avec le pourcentage d'inoculum à 10% (v/v), proches de 0.45 et 0.42 g 2,3-BD/g lactose pour W1 et PW1 respectivement à 37ºC, sous 1 atm, un pH initial 7.4 et une vitesse d'agitation de 100 rpm. En ce qui concerne l'influence du pH initial, il n'y a eu aucun changement constaté sur le rendement en 2,3-BD. Dans ce cas particulier, en présence de W1 et PW1, les rendements en 2,3-BD ont été de 0.47 et 0.44 g 2,3-BD/g lactose, respectivement à 10% (v/v) d'inoculum, à 37ºC, sous 1 atm et une vitesse d'agitation de 100 rpm. En ce qui concerne la fermentation du lactosérum à 10% (v/v) d'inoculum, à 37ºC, sous 1 atm et un pH initial de 7.4, une vitesse d'agitation de 200 rpm, un rendement maximum en 2,3-BD de 0.23 g/g lactose après 24 h a été obtenu. Ce rendement est deux fois plus faible que celui obtenu avec une vitesse d'agitation de 100 rpm après 72 h. Pour la fermentation du PW1 dans des conditions opératoires similaires, une vitesse d'agitation de 200 rpm a conduit aux mêmes effets sur le rendement en 2,3-BD.

Un nouveau lot de W (W2) et de PW (PW2) a été fermenté dans des fioles de 0.5 L puis dans un bioréacteur de 2 L (sans aération) à 10% (v/v) d'inoculum, à 37ºC, sous 1 atm, avec un pH initial de 7.4 et une vitesse d'agitation de 100 rpm. Le W2 et le PW2 ont respectivement une concentration de lactose de 51 et 47 g/L. Les deux effluents ont été fermentés directement et dilués avec de l'eau distillée pour obtenir respectivement une concentration en lactose de 31 et 34 g/L que ce soit pour les expériences menées en fiole ou en bioréacteur de 2 L. En fiole, les rendements en 2,3-BD obtenus avec les substrats W2 et PW2 dilués sont comparables à ceux issus des expériences effectuées avec les substrats W1 et PW1. En condition anaérobie, il a été constaté dans les deux cas (W1 et W2 ou PW1 ou PW2) que les rendements en 2,3-BD sont plus faibles.

Ensuite, en bioréacteur de 2 L, l'effet de l'apport d'air a été testé à 2 L/min pour étudier son impact sur la formation de 2,3-BD en présence de W2 dilué. L'addition d'air a permis d'augmenter le rendement en 2,3-BD de 135% (0.40 g, 2,3-BD/g lactose) par rapport à celui issu des expériences en bioréacteur de type fiole (0.5 L). Par comparaison, les rendements en 2,3-BD obtenus dans les fioles après 24 h à 10% (v/v) d'inoculum, à 37ºC, sous 1 atm, un pH initial de 7.4 et une vitesse d'agitation de 100 rpm sont plus faibles. Ainsi, l'utilisation d'un bioréacteur sous flux d'air, permet d'obtenir un meilleur rendement plus rapidement (diminution du temps de fermentation de 72 h à 24 h).

Par conséquent, le principal objectif de cette étude, qui était d'optimiser la fermentation de W et PW en présence d'une souche génétiquement modifiée d'*E. coli*, a été atteint. La fermentation des deux effluents a donné des rendements élevés en 2,3-BD, représentant 89 et 84% des rendements théoriques (W et PW, respectivement). De cette façon, la souche *E. coli* JFR12 peut être considérée comme une bonne candidate pour la valorisation de W et PW afin de produire du 2,3-BD.

List of references

ABSA (2017) American Biological Safety Association - Risk group Database. https://my.absa.org/tiki-index.php?page=Riskgroups. Accessed 19 December 2017

Afschar A, Rossell CV, Jonas R, Chanto AQ, Schaller K (1993) Microbial production and downstream processing of 2, 3-butanediol. J Biotechnol 27:317-329. doi: 10.1016/0168-1656(93)90094-4

Alexeeva S, Hellingwerf KJ, de Mattos MJT (2002) Quantitative assessment of oxygen availability: perceived aerobiosis and its effect on flux distribution in the respiratory chain of *Escherichia coli*. J Bacteriol 184(5):1402-1406. doi: 10.1128./JB.184.5.1402-1406.2002

Alexeeva SV (2000) Molecular physiology of responses to oxygen in *Escherichia coli*. Chapter 2 - concept of microaerobic cultivation. Dissertation Swammerdam Institute for Life Sciences. University of Amsterdam, Amsterdam, The Netherlands. ISNB 9090141863

Alpers DH, Tomkins GM (1966) Sequential transcription of the genes of the lactose operon and its regulation by protein synthesis. J Biol Chem 241(19):4434-4443

Al-Shorgani NK, Hamid AA, Yusoff WMW, Kalil MS (2013) Pre-optimization of medium for biobutanol production by a new isolate of solvent-producing *Clostridium*. BioResour 8:1420-1430

Antonopoulou G, Stamatelatou K, Venetsaneas N, Kornaros M, Lyberatos G (2008) Biohydrogen and methane production from cheese whey in a two-stage anaerobic process. Ind Eng Chem Res 47:5227-5233. doi: 10.1021/ie071622x

Anvari M and Motlagh RMS (2011) Enhancement of 2, 3-butanediol production by *Klebsiella oxytoca* PTCC 1402. J Biomed Biotechnol. doi: 10.1155/2011/636170

ATCC (2016) American Type Culture Collection - *Escherichia coli* (ATCC®27325™). https://www.atcc.org/Search_Results.aspx?dsNav=Ntk:PrimarySearch%7cescherichia+coli+ATCC+27325%7c3%7c,Ny:True,Ro:0,N:1000552&searchTerms=escherichia+coli+ATCC+27325&redir=1. Accessed 19 December 2017

Audic J-L, Chaufer B, Daufin G (2003) Non-food applications of milk components and dairy co-products: a review. Le Lait 83:417-438. doi: 10.1051/lait:2003027

Baldasso C, Barros T, Tessaro I (2011) Concentration and purification of whey proteins by ultrafiltration. Desalination 278:381-386. doi: 10.1016/j.desal.2011.05.055

Bayard R, Liu X, Benbelkacem H, Buffiere P, Gourdon R (2016) Can biomethane potential (BMP) be predicted from other variables such as biochemical composition in lignocellulosic biomass and related organic residues? BioEnergy Res 9:610-623

BCB (2017) British Cheese Board – Did you know that... http://www.cheeseboard.co.uk/facts/did_you_know_that-1. Accessed 19 December 2017

Becerra M, Cerdán ME, González-Siso MI (2015) Biobutanol from cheese whey. Microb Cell Fact 14:27. doi: 10.1186/s12934-015-0200-1

Belhamidi S, Larif M, Achatei A, Habziz S, Zouhri N, Rafiq M, Chouni S, Elhannouni F, Elmidaoui A (2015) Recovery and purification of whey with the concentration, reincorporation and reuse protein and lactose. J Mat Environ Sci 6:861-868

Berg JM, Tymoczko JL, Stryer L (2002). Biochemistry. 5th ed. New York: W H Freeman. Section 16.1, Glycolysis is an energy-conversion pathway in many organisms. Available from: https://www.ncbi.nlm.nih.gov/books/NBK22593/

Berney M, Weilenmann H-U, Ihssen J, Bassin C, Egli T (2006) Specific growth rate determines the sensitivity of *Escherichia coli* to thermal, UVA, and solar disinfection. Appl Environ Microbiol 72:2586-2593

Bertsch R (2005) Efficient production of energy, vol 401, pp 10-27. Bulletin-International Dairy Federation, Brussels

Betts J, Warr, SRC, Finka GB, Uden M, Town M, Janda JM, Baganz F, Lye GJ (2014) Impact of aeration strategies on fed-batch cell culture kinetics in a single-use 24-well miniature bioreactor. Biochem Eng J 82:105-116. doi: 10.1016/j.bej.2013.11.010

Bhandari V, Ahmod NZ, Shah HN, Gupta RS (2013) Molecular signatures for *Bacillus* species: demarcation of the *Bacillus subtilis* and *Bacillus cereus* clades in molecular terms and proposal to limit the placement of new species into the genus *Bacillus*. Int J Syst Evol Microbiol 63:2712-2726. doi:10.1099/ijs.0.048488-0

Białkowska AM (2016) Strategies for efficient and economical 2,3-butanediol production: new trends in this field. World J Microbiol Biotechnol 32:200 doi:10.1007/s11274-016-2161-x

Biebl H, Menzel K, Zeng A-P, Deckwer W-D (1999) Microbial production of 1, 3-propanediol. Appl Microbiol Biotechnol 52:289-297. doi: 10.1007/s002530051523

Black JG (2012) Microbiology: principles and explorations, 8th ed. Chapter 7, Wiley, Hoboken, p. 178-211. ISBN 9780470541098047054109 1

Blanco Londoño SA, Rodríguez Chaparro T (2012) Producción de biohidrógeno a partir de residuos mediante fermentación oscura: una revisión crítica (1993-2011). Ing Rev Chil Ing 20(3):398-411

Boundless (2016a) Classification of microorganisms by growth temperature. Boundless Microbiology. https://www.boundless.com/microbiology/textbooks/boundless-microbiology-textbook/culturing-microorganisms-6/temperature-and-microbial-growth-64/classification-of-microorganisms-by-growth-temperature-388-5509/. Accessed 19 December 2017

Boundless (2016b) Primary and secondary metabolites. Boundless Microbiology. https://www.boundless.com/microbiology/textbooks/boundless-microbiology-textbook/industrial-microbiology-17/industrial-microbiology-198/primary-and-secondary-metabolites-999-5345/. Accessed 19 December 2017

Burgos-Rubio CN, Okos MR, Wankat PC (2000) Kinetic study of the conversion of different substrates to lactic acid using *Lactobacillus bulgaricus*. Biotechnol Prog 16:305-314. doi: 10.1021/bp000022p

Canli O (2005) Physico-chemical treatment of cheese whey effluent. Dissertation, Dokuz Eylul University, Izmir (Turkey), p 122

Capper JL, Cady R, Bauman D (2009) The environmental impact of dairy production: 1944 compared with 2007. J Anim Sci 87:2160-2167. doi: 10.2527/jas.2009-1781

CAR/PL (2002) Centro de Actividad Regional para la Producción Limpia. Prevención de la contaminación en la industria láctea. Plan de Acción para el Mediterráneo. Ministerio de Medio Ambiente. Gobierno de España, p 164.

Carrascal RPU, Ochoa JAO, Cárdenas DAB (2010) Desarrollo de un estudio que conlleve a la obtención de etanol a partir del lactosuero generado por la industria láctea. II Simposio Internacional y III Nacional Agroalimentario Universidad De Córdoba (Argentina) Facultad De Ciencias Agrícolas

Casper J, Wendorff W, Thomas D (1998) Seasonal changes in protein composition of whey from commercial manufacture of caprine and ovine specialty cheeses. J Dairy Sci 81(12):3117-3122. doi:10.3168/jds.S0022-0302(98)75876-X

Castañeda Martínez T, Boucher F, Sánchez Vera E, Espinoza Ortega A (2009) La concentración de agroindustrias rurales de producción de quesos en el noroeste del Estado de México: un estudio de caracterización. Estudios sociales (Hermosillo, Son) 17(34):73-109

CDC (2017a) Centers for Disease Control and Prevention (*Escherichia coli*). http://www.cdc.gov/ecoli/general/. Accessed 19 December 2017

CDC (2017b) Centers for Disease Control and Prevention - *Enterobacter aerogenes* and *Enterobacter cloacae*. https://www.cdc.gov/drugresistance/pdf/0009.pdf and https://www.cdc.gov/drugresistance/pdf/0038c.pdf. Accessed 19 December 2017

CDC (2017c) Centers for Disease Control and Prevention - Preventing Deaths of Farm Workers in Manure Pits. http://www.cdc.gov/niosh/docs/90-103/. Accessed 19 December 2017

CDIC (2017a) Canadian Dairy Information Center - Value of Manufactured Shipments. http://www.dairyinfo.gc.ca/index_e.php?s1=dff-fcil&s2=proc-trans&s3=psdp-pvpl. Accessed 19 December 2017

CDIC (2017b) Canadian Dairy Information Center - Employment of Dairy Processing Sector http://www.dairyinfo.gc.ca/index_e.php?s1=dff-fcil&s2=proc-trans&s3=impact. Accessed 19 December 2017

CDIC (2017c) Canadian Dairy Information Center - Canadian Cheese Directory. http://cheese-fromage.agr.gc.ca/index.cfm?lang=en&menupos=1. Accessed 19 December 2017

CDIC (2017d) Canadian Dairy Information Center - World Production of Cow's Milk. http://www.dairyinfo.gc.ca/index_e.php?s1=dff-fcil&s2=farm-ferme&s3=prod&s4=glob. Accessed 19 December 2017

CDIC (2017e) Canadian Dairy Information Center - Milk Production at the Farm by Province. http://aimis-simia-cdic-ccil.agr.gc.ca/rp/index-eng.cfm?action=pR&r=234&pdctc=. Accessed 19 December 2017

CDIC (2017f) Canadian Dairy Information Center - Manufacturing of Dairy Products - Cheese. http://www.dairyinfo.gc.ca/index_e.php?s1=dff-fcil&s2=proc-trans&s3=psdp-pvpl&s4=cp-pf&page=prod_cprov. Accessed 19 December 2017

Celińska E, Grajek W (2009) Biotechnological production of 2, 3-butanediol—current state and prospects. Biotechnol Adv 27:715-725. doi: 10.1016/j.biotechadv.2009.05.002

Chan S, Jantama SS, Kanchanatawee S, Jantama K (2016) Process optimization on micro-aeration supply for high production yield of 2, 3-butanediol from maltodextrin by metabolically-engineered *Klebsiella oxytoca*. PloS ONE 11:1-20. doi: 10.1371/journal.pone.0161503

Chatzipaschali AA, Stamatis AG (2012) Biotechnological utilization with a focus on anaerobic treatment of cheese whey: current status and prospects. Energies 5(9):3492-3525. doi: 10.3390/en5093492

Chegini G, Taheri M (2013) Whey powder: process technology and physical properties. A review. Middle-East J Sci Res 13(10):1377-1387. doi: 10.5829/idosi.mejsr.2013.13.10.1239

Cherng JY, Hou TY, Shih MF, Talsma H, Hennink WE (2013) Polyurethane-based drug delivery systems Int J Pharm 450:145-162 doi:https://doi.org/10.1016/j.ijpharm.2013.04.063

Cho S, Kim T, Woo HM, Lee J, Kim Y, Um Y (2015) Enhanced 2,3-butanediol production by optimizing fermentation conditions and engineering *Klebsiella oxytoca* M1 through overexpression of acetoin reductase. PLoS ONE 10:e0138109 doi:10.1371/journal.pone.0138109

Choi E-J, Kim J-W, Kim S-J, Seo S-O, Lane S, Park Y-C, Jin Y-S, Seo J-H (2016) Enhanced production of 2,3-butanediol in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae* through optimizing ratio of glucose/galactose. Biotechnol J 11:1424-1432 doi:10.1002/biot.201600042

Chu C, Han C, Shimizu H, Wong B (2002) The effect of fructose, galactose, and glucose on the induction of β-galactosidase in *Escherichia coli*, J Exp Microbiol Immunol, 2: 1-5

Claeys W, Verraes C, Cardoen S, De Block J, Huyghebaert A, Raes K, Dewettinck K, Herman L (2014) Consumption of raw or heated milk from different species: an evaluation of the nutritional and potential health benefits. Food Control 42:188-201. doiI: 10.1016/j.foodcont.2014.01.045

Cleveland CJ, Morris CG (2013) Handbook of Energy: diagrams, charts, and tables - section 2 - biomass. In: Morris C (ed) Handbook of Energy. Elsevier, Amsterdam, pp 35-77. doi:10.1016/B978-0-08-046405-3.00002-4

COITAVC (2011) Valorización Energética: Producción de biogás mediante codigestión anaerobia de residuos/subproductos orgánicos agroindustriales. Departament of Quality and Environment of AINIA. http://www.coitavc.org. Accessed 19 December 2017

Converti A, Perego P, Del Borghi M (2003) Effect of specific oxygen uptake rate on *Enterobacter aerogenes* energetics: carbon and reduction degree balances in batch cultivations. Biotechnol Bioeng 82(3):370-377. doi: 10.1002/bit.10570

Cota-Navarro C, Carrillo-Reyes J, Davila-Vazquez G, Alatriste-Mondragón F, Razo-Flores E (2011) Continuous hydrogen and methane production in a two-stage cheese whey fermentation system. Water Sci Technol 64(2):367-374. doi: 10.2166/wst.2011.631

Cristian O (2010) Characteristics of the untreated wastewater produced by food industry. Ann Univ Oradea Fascicle Environ Prot 15:709-714

Dai J-Y, Cheng L, He Q-F, Xiu Z-L (2015) High acetoin production by a newly isolated marine *Bacillus subtilis* strain with low requirement of oxygen supply Process Biochem 50:1730-1734. doi: https://doi.org/10.1016/j.procbio.2015.07.010.

Das B, Sarkar S, Sarkar A, Bhattacharjee S, Bhattacharjee C (2015) Recovery of whey proteins and lactose from dairy waste: a step towards green waste management. Process Saf Environ Prot. doi: 10.1016/j.psep.2015.05.006

Davin-Regli A (2015) *Enterobacter aerogenes* and *Enterobacter cloacae*; versatile bacterial pathogens confronting antibiotic treatment. Front Microbiol 6:392. doi: 10.3389/fmicb.2015.00392

de Clerck E, Vanhoutte T, Hebb T, Geerinck J, Devos J, De Vos P (2004) Isolation, characterization, and identification of bacterial contaminants in semifinal gelatin extracts. Appl Environ Microbiol 70(6):3664-3672. doi: 10.1128/AEM.70.6.3664-3672.2004

De Jesus C, Elba Ruth V, Daniel S, Sharma A (2015) Biotechnological alternatives for the utilization of dairy industry waste products. Adv Biosci Biotechnol 6:223-235.

de Wit JN (2001) Lecturer's handbook on whey and whey products, 1st edn. European Whey Products Association, Brussels.

Demirel B, Yenigun O, Onay TT (2005) Anaerobic treatment of dairy wastewaters: a review. Process Biochem 40:2583-2595. doi: 10.1016/j.procbio.2004.12.015

Dragone G, Mussatto SI, Oliveira JM, Teixeira JA (2009) Characterisation of volatile compounds in an alcoholic beverage produced by whey fermentation. Food Chem 112:929-935. doi: 10.1016/j.foodchem.2008.07.005

Ecocyc (2017) *Escherichia coli* K-12 substr. MG1655: growth media search. https://ecocyc.org/ECOLI/NEW-IMAGE?object=Growth-Media. Accessed 19 December 2017

El-Qisairi AK, Qaseer HA (2002) Oxidation of ketone by palladium(II). α-Hydroxyketone synthesis catalyzed by a bimetallic palladium(II) complex. J Organomet Chem 659:50-55 doi:http://dx.doi.org/10.1016/S0022-328X(02)01691-1

Erem F, Certel M, Karakas B (2009) Identification of *Bacillus* species isolated from ropey breads both with classical methods and API identification kits. Akdeniz Univ Ziraat Fak Derg 22(2):201-210.

Ergüder T, Tezel U, Güven E, Demirer G (2001) Anaerobic biotransformation and methane generation potential of cheese whey in batch and UASB reactors. Waste Manag 21:643-650. doi: 10.1016/S0956-053X(00)00114-8

Ertem FC (2011) Improving biogas production by anaerobic digestion of different substrates: calculation of potential energy outcomes. Master dissertation in applied environmental science. Halmstad University, Halmstad, p 46.

Esmaeili A, Davison T, Wu A, Alcantara J, Jacob C (2015) PROKARYO: an illustrative and interactive computational model of the lactose operon in the bacterium *Escherichia coli*. BMC Bioinform 16:311. doi: 10.1186/s12859-015-0720-z

FAO-FAOSTAT (2017a) Emissions intensities. http://www.fao.org/faostat/en/#data/EI/visualize. Accessed 19 December 2017

FAO-FAOSTAT (2017b) Livestock primary - Production of milk. http://www.fao.org/faostat/en/#data/QL. Accessed 19 December 2017

FAO-FAOSTAT (2017c) Production, livestock primary milk, whole fresh cow. http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QL/E. Accessed 19 December 2017

FAO-FAOSTAT (2017d) Production, livestock processed cheese (all kinds). http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QP/E. Accessed 19 December 2017

FEDNA (2017) Federación Española para el Desarrollo de la Nutrición Animal - Permeato de suero. http://www.fundacionfedna.org/ingredientes_para_piensos/permeato-de-suero. Accessed 19 December 2017

Ferchichi M, Crabbe E, Gil G-H, Hintz W, Almadidy A (2005) Influence of initial pH on hydrogen production from cheese whey. J Biotechnol 120:402-409. doi: 10.1016/j.jbiotec.2005.05.017

Fernández-Gutierrez D, Veillette M, Giroir-Fendler A, Ramirez AA, Faucheux N, Heitz M (2017) Biovalorization of saccharides derived from industrial wastes such as whey: a review Rev Environ Sci Bio/Technol:1-28 doi:10.1007/s11157-016-9417-7

Fernández-Gutierrez D, Veillette M, Giroir-Fendler A, Ramirez AA, Faucheux N, Heitz M (2018) Biovalorization of glucose in four culture media and effect of the nitrogen source on fermentative alcohols production by *Escherichia coli*. Environ Technol. doi : https://doi.org/10.1080/09593330.2018.1494751

Ferrer M, Chernikova TN, Yakimov MM, Golyshin PN, Timmis KN (2003) Chaperonins govern growth of *Escherichia coli* at low temperatures. Nat Biotechnol 21(11):1266-1267. doi: 10.1038/nbt1103-1266

Fu J, Huo G, Feng L, Mao Y, Wang Z, Ma H, Chen T, Zhao X (2016) Metabolic engineering of *Bacillus subtilis* for chiral pure meso-2, 3-butanediol production. Biotechnol Biofuels 9:90. doi: 10.1186/s13068-016-0502-5

Fukiya S, Mizoguchi H, Tobe T, Mori H (2004) Extensive genomic diversity in pathogenic *Escherichia coli* and *Shigella* strains revealed by comparative genomic hybridization microarray. J Bacteriol 186(12):3911-3921. doi: 10.1128/JB.186.12.3911-3921.2004

Gao C, Zhang L, Xie Y, Hu C, Zhang Y, Li L, Wang Y, Ma C, Xu P (2013) Production of (3S)-acetoin from diacetyl by using stereoselective NADPH-dependent carbonyl reductase and glucose dehydrogenase. Bioresour Technol 137:111-115

Gao J, Xu H, Li Q-j, Feng X-h, Li S (2010) Optimization of medium for one-step fermentation of inulin extract from Jerusalem artichoke tubers using *Paenibacillus polymyxa* ZJ-9 to produce R, R-2, 3-butanediol Bioresour Technol 101:7076-7082. doi: 10.1016/j.biortech.2010.03.143

García GdJG, Fernández IM, Montenegro MMM, Fuentes AF, Cortés JdJB, Moroyoqui PG (2012) Biogás: una alternativa ecológica para la producción de energía. Ide@s CONCYTEG, 7(85):881-894. ISSN 2007-2716

García J, Santana Z, Zumalacárregui L, Quintana M, González D, Furrazola G, Cruz O (2013) Estrategias de obtención de proteínas recombinantes en *Escherichia coli* VacciMonitor 22(2):30-39. ISSN 1025-028X

Garg S, Jain A (1995) Fermentative production of 2, 3-butanediol: a review. Bioresour Technol 51:103-109. doi: 10.1016/0960-8524(94)00136-O

Ge L, Wu X, Chen J, Wu J (2011) A new method for industrial production of 2, 3-butanediol. J Biomater Nanobiotechnol 2:335-336. doi: 10.4236/jbnb.2011.23041

Gerardi MH (2003) The microbiology of anaerobic digesters, 1st edn. Wiley, New Jersey, pp 51-57. ISBN 0-471-20693-8

Gerber P, Vellinga T, Opio C, Henderson B, Steinfeld, H, FAO (2010) Greenhouse gas emissions from the dairy sector. A life cycle assessment. Food and Agricultural Organization of the United Nations: Animal Production and Health Division. Viale delle Terme di Caracalla, Rome, Italy, p 98

Ghaly A, Tango MA, Adams M (2003) Enhanced lactic acid production from cheese whey with nutrient supplement addition. Agricultural engineering international: the CIGR journal of scientific research and development manuscript FP 02 009(5):1-20. ISSN 1682-1130

Gräfje H, Körnig W, Weitz H-M, Reiß W, Steffan G, Diehl H, Bosche H, Schneider K, Kieczka H (2000) Butanediols, butenediol, and butynediol. In: Ullmann's Encyclopedia of Industrial Chemistry. Wiley-VCH Verlag GmbH and Co. KGaA. doi:10.1002/14356007.a04_455

Green EM (2011) Fermentative production of butanol—the industrial perspective. Curr Opin Biotechnol 22:1-7. doi: 10.1016/j.copbio.2011.02.004

Guignard C, Verones F, Loerincik Y, Jolliet O (2009) Environmental/ecological impact of the dairy sector: literature review on dairy products for an inventory of key issues, list of environmental initiative and influences on the dairy sector. Bulletin of the International Dairy Federation 436. 66p. ISSN 0250-5118

Guimarães PM, Teixeira JA, Domingues L (2008) Fermentation of high concentrations of lactose to ethanol by engineered flocculent *Saccharomyces cerevisiae*. Biotechnol Lett 30:1953-1958. doi: 10.1007/s10529-008-9779-1

Guimarães PM, Teixeira JA, Domingues L (2010) Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnol Adv 28:375-384. doi: 10.1016/j.biotechadv.2010.02.002

Gunasekaran S, Ko S, Xiao L (2007) Use of whey proteins for encapsulation and controlled delivery applications. J Food Eng 83:31-40. doi: 10.1016/j.jfoodeng.2006.11.001

Guo X, Guo X, Wang Y, Guo J, Wang Q, Zhang Y, Chen Y, Zhang C, Xiao D (2017) Efficient production of 2,3-butanediol from cheese whey powder (CWP) solution by *Klebsiella pneumoniae* through integrating pulsed fed-batch fermentation with a two-stage pH control strategy. Fuel 203:469-477. doi:https://doi.org/10.1016/j.fuel.2017.04.138.

Hanly TJ, Henson MA (2013) Dynamic metabolic modeling of a microaerobic yeast co-culture: predicting and optimizing ethanol production from glucose/xylose mixtures. Biotechnol Biofuels 6:44. doi: 10.1186/1754-6834-6-44

Häßler T, Schieder D, Pfaller R, Faulstich M, Sieber V (2012) Enhanced fed-batch fermentation of 2, 3-butanediol by *Paenibacillus polymyxa* DSM 365. Bioresour Technol 124:237-244. doi: 10.1016/j.biortech.2012.08.047

Hazeena SH, Pandey A, Binod P (2016) Evaluation of oil palm front hydrolysate as a novel substrate for 2, 3-butanediol production using a novel isolate *Enterobacter cloacae* SG1. Renew Energy 98:216-220. doi: 10.1016/j.renene.2016.02.030

Hernández-Rojas M, Vélez-Ruíz J (2014) Suero de leche y su aplicación en la elaboración de alimentos funcionales. Temas Sel Ing Aliment 8(2):13-22

Hespell R (1996) Fermentation of xylan, corn fiber, or sugars to acetoin and butanediol by *Bacillus polymyxa* strains. Curr Microbiol 32:291-296

Hirsch S, Hartmann M (2014) Persistence of firm-level profitability in the European dairy processing industry. Agric Econ 45:53-63. doi: 10.1111/agec.12129

Holladay JD, Hu J, King DL, Wang Y (2009) An overview of hydrogen production technologies. Catal Today 139:244-260. doi: 10.1016/j.cattod.2008.08.039

Hugenholtz J, Kleerebezem M (1999) Metabolic engineering of lactic acid bacteria: overview of the approaches and results of pathway rerouting involved in food fermentations. Curr Opin Biotechnol 10:492-497

iGEM (2017) International Genetically Engineered Machine - Safety/Risk group guide. http://2014.igem.org/Safety/Risk_Group_Guide. Accessed 19 December 2017

INTI (2017) (Instituto Nacional de Tecnología Industrial). Whey proteins concentrate in dairy industries. https://www.inti.gob.ar/lacteos/pdf/PESKO/1.pdf. Accessed 19 December 2017

Ji X-J, Huang H, Du J, Zhu J-G, Ren L-J, Hu N, Li S (2009a) Enhanced 2, 3-butanediol production by *Klebsiella oxytoca* using a two-stage agitation speed control strategy. Bioresour Technol 100:3410-3414. doi: 10.1016/j.biortech.2009.02.031

Ji X-J, Huang H, Du J, Zhu J-G, Ren L-J, Li S, Nie Z-K (2009b) Development of an industrial medium for economical 2, 3-butanediol production through co-fermentation of glucose and xylose by *Klebsiella oxytoca*. Bioresour Technol 100:5214-5218. doi: 10.1016/j.biortech.2009.05.036

Ji X-J, Huang H, Ouyang P-K (2011) Microbial 2, 3-butanediol production: a state-of-the-art review. Biotechnol Adv 29:351-364. doi: 10.1016/j.biotechadv.2011.01.007

Ji X-J, Huang H, Zhu J-G, Ren L-J, Nie Z-K, Du J, Li S (2010) Engineering *Klebsiella oxytoca* for efficient 2, 3-butanediol production through insertional inactivation of acetaldehyde dehydrogenase gene. Appl Microbiol Biotechnol 85:1751-1758. doi: 10.1007/s00253-009-2222-2

Ji X-J, Liu LG, Shen MQ, Nie ZK, Tong YJ, Huang H (2015) Constructing a synthetic metabolic pathway in *Escherichia coli* to produce the enantiomerically pure (R, R)-2, 3-butanediol. Biotechnol Bioeng 112(5):1056-1059. doi: 10.1002/bit.25512

Jiang Y, Liu W, Zou H, Cheng T, Tian N, Xian M (2014) Microbial production of short chain diols. Microb Cell Fact 13:165. doi: 10.1186/s12934-014-0165-5

Jiang L-Q, Fang Z, Zhao Z-L, He F, Li H-B (2015) 2,3-butanediol and acetoin production from enzymatic hydrolysate of ionic liquid-pretreated cellulose by *Paenibacillus polymyxa*. Bioresour 10(1), 1318-1329.

Jiayang Q, Zijun X, Cuiqing M, Nengzhong X, Peihai L, Ping X (2006) Production of 2, 3-butanediol by *Klebsiella pneumoniae* using glucose and ammonium phosphate. Chin J Chem Eng 14(1):132-136. doi: 10.1016/S1004-9541(06)60050-5

Jin Q, Yuan Z, Xu J, Wang Y, Shen Y, Lu W, Wang J, Liu H, Yang J, Yang F, Zhang X, Zhang J, Yang G, Wu H, Qu D, Dong J, Sun L, Xue Y, Zhao A, Gao Y, Zhu J, Kan B, Ding K, Chen S, Cheng H, Yao Z, He B, Chen R, Ma D, Qiang B, Wen Y, Hou Y, Yu J (2002) Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. Nucleic Acids Res 30(20):4432-4441. doi: 10.1093/nar/gkf566

Jung M-Y, Ng CY, Song H, Lee J, Oh M-K (2012) Deletion of lactate dehydrogenase in *Enterobacter aerogenes* to enhance 2,3-butanediol production. Appl Microbiol Biotechnol 95:461-469 doi:10.1007/s00253-012-3883-9

Jurado E, Camacho F, Luzon G, Vicaria J (2002) A new kinetic model proposed for enzymatic hydrolysis of lactose by a β-galactosidase from *Kluyveromyces fragilis*. Enzyme Microbial Technol 31:300-309. doi: 10.1016/S0141-0229(02)00107-2

Kandasamy V, Liu J, Dantoft SH, Solem C, Jensen PR (2016) Synthesis of (3R)-acetoin and 2,3-butanediol isomers by metabolically engineered *Lactococcus lactis*. Sci Rep 6:36769. doi:10.1038/srep36769

Karadag D, Köroğlu OE, Ozkaya B, Cakmakci M (2015) A review on anaerobic biofilm reactors for the treatment of dairy industry wastewater. Process Biochem 50:262-271. doi: 10.1016/j.procbio.2014.11.005

Keri Marshall N (2004) Therapeutic applications of whey protein. Altern Med Rev 9(2):136-156

Khajavi SH, Ota S, Nakazawa R, Kimura Y, Adachi S (2006) Hydrolysis kinetics of trisaccharides consisting of glucose, galactose, and fructose residues in subcritical water. Biotechnol Prog 22(5):1321-1326. doi: 10.1021/bp060086I

Khanal SK (2008) Anaerobic biotechnology for bioenergy production. Chapter 1 - overview of anaerobic biotechnology. In: Khanal SK (ed) Principles and application, 1st edn. Willey, Ames, pp 1-28. doi:10-1002/9780813804545.ch1

Kim T, Cho S, Lee S-M, Woo HM, Lee J, Um Y, Seo J-H (2016) High production of 2,3-butanediol (2,3-BD) by *Raoultella ornithinolytica* B6 via optimizing fermentation conditions and overexpressing 2,3-BD synthesis genes. PLoS ONE 11(10): e0165076. doi:10.1371/journal.pone.0165076

Köpke M, Mihalcea C, Liew FM, Tizard JH, Ali MS, Conolly JJ, Al-Sinawi B, Simpson SD (2011) 2, 3-Butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. Appl Environ Microbiol 77(15):5467-5475. doi: 10.1128/AEM.00355-11

Koutinas M, Menelaou M, Nicolaou EN (2014) Development of a hybrid fermentation–enzymatic bioprocess for the production of ethyl lactate from dairy waste. Bioresour Technol 165:343-349. doi: 10.1016/j.biortech.2014.03.053

Krämer R (2010) Bacterial stimulus perception and signal transduction: response to osmotic stress. Chem Record 10:217-229. doi: 10.1002/tcr.201000005

Lara ÁR (2011) Producción de proteínas recombinantes en *Escherichia coli*. Rev Mex Ing Quím 10(2):209-223. ISSN 1665-2738

Lazar Z, Gamboa-Meléndez H, Crutz-Coq A-M, Neuvéglise C, Nicaud J-M (2015). Awakening the endogenous Leloir pathway for efficient galactose utilization by *Yarrowia lipolytica*. Biotechnol Biofuels 8:1.

Lee HK, Maddox IS (1986) Continuous production of 2,3-butanediol from whey permeate using *Klebsiella pneumoniae* immobilized in calcium alginate. Enzyme Microb Technol 8:409-411 doi:http://dx.doi.org/10.1016/0141-0229(86)90147-X

Lee J, Jang Y-S, Choi SJ, Im JA, Song H, Cho JH, Papoutsakis ET, Bennett GN, Lee SY (2012) Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. Appl Environl Microbiol 78(5):1416-1423. doi: 10.1128/AEM.06382-11

Lehtomäki A, Huttunen S, Rintala J (2007) Laboratory investigations on co-digestion of energy crops and crop residues with cow manure for methane production: effect of crop to manure ratio. Resour Conserv Recycl 51:591-609. doi: 10.1016/j.resconrec.2006.11.004

Levine RL, Moskovitz J, Stadtman ER (2000) Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. IUBMB Life 50:301-307. doi: 10.1080/713803735

Li J, Wang W, Ma Y, Zeng A-P (2013) Medium optimization and proteome analysis of (R, R)-2, 3-butanediol production by *Paenibacillus polymyxa* ATCC 12321. Appl Microbiol Biotechnol 97:585-597. doi: 10.1007/s00253-012-4331-6

Li L, Li L, Li K, Wang Y, Chen C, Xu Y, Zhang L, Han B, Gao C, Tao F, Ma C, Xu P (2015) Metabolic engineering of *Enterobacter cloacae* for high-yield production of enantiopure (2R, 3R)-2, 3-butanediol from lignocellulose-derived sugars. Metab Eng 28:19-27. doi: 10.1016/j.ymben.2014.11.010

Liang Q, Zhang H, Li S, Qi Q (2011) Construction of stress-induced metabolic pathway from glucose to 1, 3-propanediol in *Escherichia coli*. Appl Microbiol Biotechnol 89:57-62. doi: 10.1007/s00253-010-2853-3

Lievore P, Simões DR, Silva KM, Drunkler NL, Barana AC, Nogueira A, Demiate IM (2015) Chemical characterisation and application of acid whey in fermented milk. J Food Sci Technol 52(4):2083-2092. doi: 10.1007/s13197-013-1244-z

Lu M, Lee S, Kim B, Park C, Oh M, Park K, Lee SY, Lee J (2012) Identification of factors regulating *Escherichia coli* 2, 3-butanediol production by continuous culture and metabolic flux analysis. J Microbiol Biotechnol 22(5):659-667. doi: 10.4014/jmb.1112.12018

Luo Y, Zhang T, Wu H (2014) The transport and mediation mechanisms of the common sugars in *Escherichia coli*. Biotechnol Adv 32:905-919. doi:http://dx.doi.org/10.1016/j.biotechadv.2014.04.009

Ma C, Wang A, Qin J, Li L, Ai X, Jiang T, Tang H, Xu P (2009) Enhanced 2, 3-butanediol production by *Klebsiella pneumoniae* SDM. Appl Microbiol Biotechnol 82:49-57. doi: 10.1007/s00253-008-1732-7

Madureira AR, Pereira CI, Gomes AM, Pintado ME, Malcata FX (2007) Bovine whey proteins–overview on their main biological properties. Food Res Int 40:1197-1211. doi: 10.1016/j.foodres.2007.07.005

Magee RJ, Kosaric N (1987) The microbial production of 2, 3-butanediol. Adv Appl Microbiol 32:89-161. doi: 10.1016/S0065-2164(08)70079-0

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. 1st edition. Pages 545. Cold Spring Harbor Laboratory Cold Spring Harbor, NY (USA). ISBN: 0879691360

Martins RC, Quinta-Ferreira RM (2010) Final remediation of post-biological treated milk whey wastewater by ozone. Int J Chem Reactor Eng 8:A142. doi: 10.2202/1542-6580.2310

Matlock BC, Beringer RW, Ash DL, Allen MW, Page AF (2017) Analyzing differences in bacterial optical density measurements between spectrophotometers. https://tools.thermofisher.com/content/sfs/posters/nanodroptryposter.pdf. Accessed 19 December 2017

Mazumdar S, Lee J, Oh M-K (2013) Microbial production of 2, 3 butanediol from seaweed hydrolysate using metabolically engineered *Escherichia coli*. Bioresour Technol 136:329-336. doi: 10.1016/j.biortech.2013.03.013

Mekonnen M, Hoekstra A (2010) The green, blue and grey water footprint of farm animals and animal products. Value of water research report series no 48 (UNESCO-IHE, Delft, The Netherlands)

Minhalma M, Magueijo V, Queiroz DP, de Pinho MN (2007) Optimization of "Serpa" cheese whey nanofiltration for effluent minimization and by-products recovery. J Environ Manage 82:200-206. doi: 10.1016/j.jenvman.2005.12.011

Miranda G, Hazé G, Scanff P, Pelissier J (1989) Hydrolysis of α-lactalbumin by chymosin and pepsin. Effect of conformation and pH. Le Lait 69(6):451-459. doi: 10.1051/lait:1989630

Miranda O, Ponce I, Fonseca P, Cutiño M, Díaz R, Cedeño C (2009) Características físico-químicas de sueros de queso dulce y ácido producidos en el combinado de quesos de Bayamo. Rev Cubana de Aliment Nutr 19(1):21-25

Missouri Department of Natural Resources – Maximum dissolved oxygen concentration saturation table. https://dnr.mo.gov/env/esp/wqm/DOSaturationTable.htm. Acceded on 19 December 2017

Moral R, Moreno-Caselles J, Perez-Murcia M, Perez-Espinosa A, Rufete B, Paredes C (2005) Characterisation of the organic matter pool in manures. Bioresour Technol 96:153-158. doi: 10.1016/j.biortech.2004.05.003

Muehlhoff E, Bennett A, McMahon D (eds) (2013) Milk and dairy products in human nutrition. Food and Agriculture Organization of the United Nations (FAO), Rome, pp 376. ISBN 978-92-5-107863-1

Mukhopadhyay R, Talukdar D, Chatterjee BP, Guha AK (2003) Whey processing with chitosan and isolation of lactose. Process Biochem 39:381-385. doi: 10.1016/S0032-9592(03)00126-2

Nakashimada Y, Kanai K, Nishio N (1998) Optimization of dilution rate, pH and oxygen supply on optical purity of 2, 3-butanediol produced by *Paenibacillus polymyxa* in chemostat culture. Biotechnol Lett 20(12):1133-1138. doi: 10.1023/A:1005324403186

Nakashimada Y, Marwoto B, Kashiwamura T, Kakizono T, Nishio N (2000) Enhanced 2, 3-butanediol production by addition of acetic acid in *Paenibacillus polymyxa*. J Biosci Bioeng 90(6):661-664. doi: 10.1016/S1389-1723(00)90013-6

Narayanan N, Roychoudhary P, Srivastava A (2004) Lactic acid fermentation and its product polymerization. Electron J Biotechnol 7(2): 167-179. doi: 10.4067/S0717-34582004000200008

Neri DF, Balcão VM, Carneiro-da-Cunha MG, Carvalho Jr LB, Teixeira JA (2008) Immobilization of β-galactosidase from *Kluyveromyces lactis* onto a polysiloxane–polyvinyl alcohol magnetic (mPOS–PVA) composite for lactose hydrolysis. Catal Commun 9:2334-2339. doi: 10.1016/j.catcom.2008.05.022

Nielsen DR, Yoon S-H, Yuan CJ, Prather KLJ (2010) Metabolic engineering of acetoin and meso-2, 3-butanediol biosynthesis in *E. coli*. Biotechnol J 5:274-284 doi:10.1002/biot.200900279

Noumi ES, Blin J, Valette J, Rousset P (2015) Combined effect of pyrolysis pressure and temperature on the yield and CO_2 gasification Reactivity of acacia wood in macro-TG. Energy Fuels 29:7301-7308 doi:10.1021/acs.energyfuels.5b01454

Ochoa Gómez J, Gil Río S, Río Pérez F, Lorenzo Ibarreta L, Diñeiro García C, Roncal Martínez T (2017) Method for manufacturing 2,3-butanediol. Patent: EP3173398 (A1)

Okonkwo C, Ujor V, Mishra P, Ezeji T (2017) Process development for enhanced 2,3-butanediol production by *Paenibacillus polymyxa* DSM 365. Fermentation 3:18 doi:10.3390/fermentation3020018

Omil F, Garrido JM, Arrojo B, Méndez R (2003) Anaerobic filter reactor performance for the treatment of complex dairy wastewater at industrial scale. Water Res 37:4099-4108. doi: 10.1016/S0043-1354(03)00346-4

Oomori T, Khajavi SH, Kimura Y, Adachi S, Matsuno R (2004) Hydrolysis of disaccharides containing glucose residue in subcritical water. Biochem Eng J 18:143-147. doi: 10.1016/j.bej.2003.08.002

Padín González C, Díaz Fernández M (2009) Fermentación alcohólica del lactosuero por *Kluyveromyces marxianus* y solventes orgánicos como extractantes. Rev Soc Ven Microbiol 29:110-116. ISSN 1315-2556

Panesar PS, Kennedy JF, Gandhi DN, Bunko K (2007) Bioutilisation of whey for lactic acid production. Food Chem 105:1-14. doi: 10.1016/j.foodchem.2007.03.035

Panesar PS, Kennedy JF, Knill CJ, Kosseva M (2010) Production of L (+) lactic acid using *Lactobacillus casei* from whey. Braz Arch Biol Technol 53(1):219-226. doi: 10.1590/S1516-89132010000100027

Pardee AB, Jacob F, Monod J (1959) The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *E. coli*. J Mol Biol 1:165-178

Parra Huertas RA (2009) Whey: importance in the food industry Rev Fac Nac Agron Medellín 62(1):4967-4982

Patel M, Crank M, Dornburg V, Hermann B, Roes L, Hüsing B, Overbeek L, Terragni F, Recchia E (2006) The BREW project: medium and long-term opportunities and risks of the biotechnological production of bulk chemicals from renewable resources–the potential of white biotechnology. Department of Science, Technology and Society (STS)/Copernicus Institute, Utrecht (Netherlands). ISSN 0022-1155

Patel S (2015) Emerging trends in nutraceutical applications of whey protein and its derivatives. J Food Sci Technol 52:6847-6858. doi: 10.1007/s13197-015-1894-0

Pennington JA, VanDevender K, Jennings, JA, Arkansas-University (2009) Nutrient and fertilizer value of dairy manure. https://www.uaex.edu/publications/PDF/FSA-4017.pdf. Accessed 19 December 2017

Perego P, Converti A, Del Borghi A, Canepa P (2000) 2,3-Butanediol production by *Enterobacter aerogenes*: selection of the optimal conditions and application to food industry residues. Bioprocess Eng 23:613-620. doi: doi:10.1007/s004490000210

Perego P, Converti A, Del Borghi M (2003) Effects of temperature, inoculum size and starch hydrolyzate concentration on butanediol production by *Bacillus licheniformis*. Bioresour Technol 89:125-131. doi: 10.1016/S0960-8524(03)00063-4

Pereira-Rodríguez Á, Fernández-Leiro R, González-Siso MI, Cerdán ME, Becerra M, Sanz-Aparicio J (2012) Structural basis of specificity in tetrameric *Kluyveromyces lactis* β-galactosidase. J Struct Biol 177:392-401. doi: 10.1016/j.jsb.2011.11.031

Pescuma M, de Valdez GF, Mozzi F (2015) Whey-derived valuable products obtained by microbial fermentation. Appl Microbiol Biotechnol 99:6183-6196. doi: 10.1007/s00253-015-6766-z

Pescuma M, Hébert EM, Mozzi F, de Valdez GF (2008) Whey fermentation by thermophilic lactic acid bacteria: evolution of carbohydrates and protein content. Food Microbiol 25:442-451. doi: 10.1016/j.fm.2008.01.007

Petrov K, Petrova P (2009) High production of 2,3-butanediol from glycerol by *Klebsiella pneumoniae* G31. Appl Microbiol Biotechnol 84:659-665. doi: 10.1007/s00253-009-2004-x

PHAC – Public Health Agency of Canada (2017). Pathogen safety data sheets and risk assessment. http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php. Accessed 19 December 2017

Pintado M, Macedo A, Malcata F (2001) Review: technology, chemistry and microbiology of whey cheeses. Food Sci Technol Int, 7(2), 105–116. doi: 10.1106/TY4X-QX5U-5X5D-TYE8

Portnoy VA, Herrgård MJ, Palsson BØ (2008) Aerobic fermentation of D-glucose by an evolved cytochrome oxidase-deficient *Escherichia coli* strain. Appl Environ Microbiol 74(24):7561-7569. doi: 10.1128/AEM.00880-08

Prakash G (2015) A framework for sustainable food supply chain: reflections from the indian dairy producers. In: Kachitvichyanukul V, Sethanan K, Golinska-Dawson P (eds) Toward sustainable operations of supply chain and logistics systems. Springer, Heidelberg, pp 161-182. doi:10.1007/978-3-319-19006-8

Prazeres AR, Carvalho F, Rivas J (2012) Cheese whey management: a review. J Environ Manage 110:48-68. doi: 10.1016/j.jenvman.2012.05.018

Priya A, Dureja P, Talukdar P, Rathi R, Lal B, Sarma PM (2016) Microbial production of 2, 3-butanediol through a two-stage pH and agitation strategy in 150 L bioreactor. Biochem Eng J 105:159-167. doi: 10.1016/j.bej.2015.09.016

Public Health Agency of Canada (PHAC) (2017). *Klebsiella* spp. Pathogen safety data sheet - Infectious substances. http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/klebsiella-eng.php. Accessed 19 December 2017

Qi G, Kang Y, Li L, Xiao A, Zhang S, Wen Z, Xu D, Chen S (2014) Deletion of meso-2,3-butanediol dehydrogenase gene *bud C* for enhanced D-2,3-butanediol production in *Bacillus licheniformis* Biotechnol Biofuels 7:16 doi:10.1186/1754-6834-7-16

Qiu Y, Zhang J, Li L, Wen Z, Nomura CT, Wu S, Chen S (2016) Engineering *Bacillus licheniformis* for the production of meso-2,3-butanediol Biotechnol Biofuels 9:117 doi:10.1186/s13068-016-0522-1

Quigley T (2008) Monitoring the growth of *E. coli* with light scattering using the synergy™ 4 multi-mode microplate reader with hybrid technology. http://mktg.biotek.com/TekTalk/2008/0208/tek_corner.htm. Accessed 19 December 2017

Qureshi N, Maddox I (2005) Reduction in butanol inhibition by perstraction: utilization of concentrated lactose/whey permeate by *Clostridium acetobutylicum* to enhance butanol fermentation economics. Food Bioprod Process 83:43-52. doi: 10.1205/fbp.04163

Raganati F, Olivieri G, Procentese A, Russo M, Salatino P, Marzocchella A (2013) Butanol production by bioconversion of cheese whey in a continuous packed bed reactor. Bioresour Technol 138:259-265. doi: 10.1016/j.biortech.2013.03.180

Ramirez C, Patel M, Blok K (2006) From fluid milk to milk powder: energy use and energy efficiency in the European dairy industry. Energy 31:1984-2004. doi: 10.1016/j.energy.2005.10.014

Rebouillat S, Ortega-Requena S (2015) Potential applications of milk fractions and valorization of dairy by-products: a review of the state-of-the-art available data, outlining the innovation potential from a bigger data standpoint. J Biomater Nanobiotechnol 6:176-203. doi: 10.4236/jbnb.2015.63018

Ridoutt BG, Pfister S (2010) A revised approach to water footprinting to make transparent the impacts of consumption and production on global freshwater scarcity. Glob Environ Change 20:113-120. doi: 10.1016/j.gloenvcha.2009.08.003

Roncal T, Cadiermo U, Torrecilla J, García Jaca J, Garmendia I, Azpeitia M, Ochoa-Gómez J (2010) La biotecnología en el sector químico (II). Nuevas tendencias en la producción industrial de productos químicos básicos. Ing Quím 478:152-160

Roncal T, Cadierno U, Torrecilla J, García J, Garmendia I, Azpeitia M, Ochoa J (2009) Biotecnología-La biotecnología en el sector químico (I)-Producción industrial de productos químicos básicos. Ing Quím 476:120-123

Rorabacher DB (1991) Statistical treatment for rejection of deviant values: critical values of Dixon's" Q" parameter and related subrange ratios at the 95% confidence level. Anal Chem 63:139-146

Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges. Front Microbiol 5:172 doi:10.3389/fmicb.2014.00172

Rosenberg E, Gophna U (2011) Beneficial microorganisms in multicellular life forms, 1st edn. Springer Science & Business Media, Heidelberg, p 348.

Saddoud A, Hassaïri I, Sayadi S. 2007. Anaerobic membrane reactor with phase separation for the treatment of cheese whey. Bioresour Technol 98:2102-2108. doi: 10.1016/j.biortech.2006.08.013

Saggar S, Bolan N, Bhandral R, Hedley C, Luo J (2004) A review of emissions of methane, ammonia, and nitrous oxide from animal excreta deposition and farm effluent application in grazed pastures. N Z J Agric Res 47:513-544. doi: 10.1080/00288233.2004.9513618

Saha B, Bothast R (1999) Production of 2, 3-butanediol by newly isolated *Enterobacter cloacae*. Appl Microbiol Biotechnol 52:321-326. doi: 10.1007/s002530051526

Santo Pereira R, Dias Cordeiro V, Barbosa Ferreira-Machado A, Alves Resende J, Netto Bastos A, Quinet de Andrade Bastos L, Quinet de Andrade Bastos V, Villela Bastos R, da Silva VL, Galuppo Diniz C (2016) Physiological and molecular characteristics of carbapenem resistance in *Klebsiella pneumoniae* and *Enterobacter aerogenes*. J Infect Dev Ctries 10:592-599. doi: 10.3855/jidc.6821

Sauer U, Canonaco F, Heri S, Perrenoud A, Fischer E (2004) The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. J Biol Chem 279:6613-6619 doi:10.1074/jbc.M311657200

Savakis PE, Angermayr SA, Hellingwerf KJ (2013) Synthesis of 2, 3-butanediol by *Synechocystis* sp. PCC6803 via heterologous expression of a catabolic pathway from lactic acid-and *enterobacteria*. Metab Eng 20:121-130. doi: 10.1016/j.ymben.2013.09.008

Saxena R, Anand P, Saran S, Isar J (2009) Microbial production of 1, 3-propanediol: recent developments and emerging opportunities. Biotechnol Adv 27:895-913. doi: 10.1016/j.biotechadv.2009.07.003

Schaafsma G (2008) Lactose and lactose derivatives as bioactive ingredients in human nutrition. Int Dairy J 18:458-465. doi: 10.1016/j.idairyj.2007.11.013

Sezonov G, Joseleau-Petit D, D'Ari R (2007) *Escherichia coli* physiology in Luria-Bertani broth. J Bacteriol 189:8746-8749 doi:10.1128/JB.01368-07

Shi L, Gao S, Yu Y, Yang H (2014) Microbial production of 2, 3-butanediol by a newly-isolated strain of *Serratia marcescens*. Biotechnol Lett 36:969-973. doi: 10.1007/s10529-013-1433-x

Shin H-D, Yoon S-H, Wu J, Rutter C, Kim S-W, Chen RR (2012) High-yield production of meso-2,3-butanediol from cellodextrin by engineered *E. coli* biocatalysts. Bioresour Technol 118:367-373. doi: 10.1016/j.biortech.2012.04.100

SigmaAldrich (2017). SigmaAldrich product directory home. http://www.sigmaaldrich.com/technical-service-home/product-catalog.html. Accessed 19 December 2017

Sikdar MSI, Kim J-S (2010) Expression of a gene encoding acetolactate synthase from rice complements two ilvH mutants in *Escherichia coli*. Aust J Crop Sci 4(6):430-436

Silveira M, Berbert-Molina M, Prata A, Schmidell W (1998) Production of 2,3-butanediol from sucrose by *Klebsiella pneumoniae* NRRL B199 in batch and fed-batch reactors. Braz Arch Biol Technol 41(3):329-334. doi: 10.1590/S1516-89131998000300009

Silveira W, Passos F, Mantovani H, Passos F (2005) Ethanol production from cheese whey permeate by *Kluyveromyces marxianus* UFV-3: a flux analysis of oxido-reductive metabolism as a function of lactose concentration and oxygen levels. Enzyme Microbial Technol 36:930-936. doi: 10.1016/j.enzmictec.2005.01.018

Sinha P, Pandey A (2011) An evaluative report and challenges for fermentative biohydrogen production. Int J Hydrog Energy 36:7460-7478. doi: 10.1016/j.ijhydene.2011.03.077

Sinha P, Roy S, Das D (2015) Role of formate hydrogen lyase complex in hydrogen production in facultative anaerobes. Int J Hydrog Energy 40:8806-8815. doi: 10.1016/j.ijhydene.2015.05.076

SM (2017) Science Museum - How are proteins made? http://www.sciencemuseum.org.uk/whoami/findoutmore/yourbody/whatdoyourcellsdo/howdocellsmakeproteins/howareproteinsmade. Accessed 19 December 2017

Smith ST, Metzger L, Drake MA (2016) Evaluation of whey, milk, and delactosed permeates as salt substitutes. J Dairy Sci 99:8687-8698 doi:10.3168/jds.2016-10904.

Smithers GW (2015) Whey-ing up the options–Yesterday, today and tomorrow. Int Dairy J 48:2-14. doi: 10.1016/j.idairyj.2015.01.011

Somerville GA, Proctor RA (2013) Cultivation conditions and the diffusion of oxygen into culture media: the rationale for the flask-to-medium ratio in microbiology. BMC Microbiol 13:9 doi:10.1186/1471-2180-13-9

Spalatelu C (2012) Biotechnological valorisation of whey. Innov Roman Food Biotechnol 10:1-8. ISSN 1843-6099

Statistic-Canada (2006) A Geographical profile of livestock manure production in Canada. Lots of manure, especially from cattle – how much manure does each animal produce? http://www.statcan.gc.ca/pub/16-002-x/2008004/article/10751-eng.htm. Accessed 19 December 2017

Sutton S (2011) Measurement of microbial cells by optical density. J Validation Technol 17(1):46-49

Tang X, Tan Y, Zhu H, Zhao K, Shen W (2009) Microbial conversion of glycerol to 1,3-propanediol by an engineered strain of *Escherichia coli*. Appl Environ Microbiol 75:1628-1634. doi: 10.1128/AEM.02376-08

Tian Y, Fan Y, Zhao X, Zhang J, Yang L, Liu J (2014) Optimization of fermentation medium for acetoin production by *Bacillus subtilis* SF4-3 using statistical methods. Prep Biochem Biotechnol 44:529-543. doi:10.1080/10826068.2013.835731

Tikariha A, Sahu O (2014) Study of characteristics and treatments of dairy industry waste water. J Appl Environ Microbiol 2:16-22. doi: 10.12691/jaem-2-1-4

Tiso M, Schechter AN (2015) Nitrate reduction to nitrite, nitric oxide and ammonia by gut bacteria under physiological conditions. PLoS ONE 10:e0127490. doi:10.1371/journal.pone.0127490

Todar K (2017) Todar's Online Textbook of Bacteriology. University of Wisconsin-Madison Department of Bacteriology. https:// http://textbookofbacteriology.net/nutgro.html. Accessed 19 December 2017

Tong Y-J, Ji X-J, Shen M-Q, Liu L-G, Nie Z-K, Huang H (2016) Constructing a synthetic constitutive metabolic pathway in *Escherichia coli* for (R, R)-2,3-butanediol production. Appl Microbiol Biotechnol 100:637-647 doi:10.1007/s00253-015-7013-3

Trivedi MK, Branton A, Trivedi D, Gangwar M, S J (2015a) Antimicrobial susceptibility, biochemical characterization and molecular typing of biofield treated *Klebsiella pneumoniae*. J Health Med Inf 6: 206 doi:104172/2157-74201000206

Trivedi MK, Patil S, Shettigar H, Bairwa K, Jana S (2015b) Phenotypic and biotypic characterization of *Klebsiella oxytoca*: An impact of biofield treatment. J Microb Biochem Technol 7(4): 202-205. doi: 10.4172/1948-5948.1000205

Tsakali E, Petrotos K, D'Allessandro A, Goulas P (2010) A review on whey composition and the methods used for its utilization for food and pharmaceutical products. In: 6th international conference on simulation and modelling in the food and bio-industry FOODSIM, research Centre, Braganca (Portugal) 24-26 June.

Türkmenoglu S (2006) Organic acids production from cheese-whey. Dissertation. Middle East Technical University, Ankara (Turkey)

Ui S, Okajima Y, Mimura A, Kanai H, Kudo T (1997) Molecular generation of an *Escherichia coli* strain producing only the meso-isomer of 2, 3-butanediol J Ferment Bioeng 84:185-189. doi: 10.1016/S0922-338X(97)82052-1

Ukpai P, Nnabuchi M (2012) Comparative study of biogas production from cow dung, cow pea and cassava peeling using 45 L biogas digester. Adv Appl Sci Res 3:1864-1869

UNAC (2017) Universidad Nacional del Callao (Perú) - metabolismo secundario. http://www.unac.edu.pe/documentos/organizacion/vri/cdcitra/Informes_Finales_Investigacion/Octubre_2011/IF_DECHECO%20EGUSQUIZA_FIPA/CAPITULO%20N%BA%2011.pdf. Accessed 19 December 2017

United-Nations (2006) Rearing cattle produce more greenhouse gases than driving cars, UN report warns. http://www.un.org/apps/news/story.asp?newsID=20772#.VRBIJtKG8UV. Accessed 19 December 2017

USDairy (Innovation center for USDairy) (2017). Coproducts of milk and whey processing. http://www.usdairy.com/~/media/usd/public/coproducts_milk_report.pdf. Accessed 19 December 2017

USDE (U.S. Department of Energy) (2017) - Lower and higher heating values of fuels. http://hydrogen.pnl.gov/tools/lower-and-higher-heating-values-fuels. Accessed 19 December 2017

USDEC (United States Dairy Export Council) (2017) U.S. Whey and milk permeate. http://www.thinkusadairy.org/assets/Documents/Customer%20Site/C1-discover%20US%20Dairy/C1.3-Products/US%20Whey-Milk-Permeate-Overview.pdf. Accessed 19 December 2017.

USEPA (U.S. Environmental Protection Agency) (2012). NPDES Permit Writers' Manual for Concentrated Animal Feeding Operations (CAFOs). Chapter 6: Protocols for Land Application of Manure Nutrients. https://www3.epa.gov/npdes/pubs/cafo_permitmanual_chapter6.pdf. Accessed 19 December 2017

Van Houdt R, Aertsen A, Michiels CW (2007) Quorum-sensing-dependent switch to butanediol fermentation prevents lethal medium acidification in *Aeromonas hydrophila* AH-1N. Res Microbiol 158:379-385. doi: 10.1016/j.resmic.2006.11.015

Venetsaneas N, Antonopoulou G, Stamatelatou K, Kornaros M, Lyberatos G (2009) Using cheese whey for hydrogen and methane generation in a two-stage continuous process with alternative pH controlling approaches. Bioresour Technol 100:3713-3717. doi: 10.1016/j.biortech.2009.01.025

Venkatesh K, Okos M, Wankat P (1993) Kinetic model of growth and lactic acid production from lactose by *Lactobacillus bulgaricus*. Process Biochem 28:231-241

Vieira DC, Lima LN, Mendes AA, Adriano WS, Giordano RC, Giordano RL, Tardioli PW (2013) Hydrolysis of lactose in whole milk catalyzed by β-galactosidase from *Kluyveromyces*

fragilis immobilized on chitosan-based matrix. Biochem Eng J 81:54-64. doi: 10.1016/j.bej.2013.10.007

Vijayakumar J, Aravindan R, Viruthagiri T (2008) Recent trends in the production, purification and application of lactic acid. Chem Biochem Eng Q 22:245-264

Vishwakarma S. (2014) Bioconversion of whey to 2, 3-butanediol using *Klebsiella oxytoca* NRRL-13-199. Indian J Biotechnol 13:236-240.

Vlahović B, Popović-Vranješ A, Mugoša I (2014) International cheese market-current state and perspective. Econ Insights Trends Chall 3(1):35-43

Voloch M, Jansen N, Ladish M, Tsao G, Narayan R, Rodwell V (1985) 2,3-Butanediol. In: Blanch HW, Drew S, Wang DC (eds). Comprehensive biotechnology: the principles, applications and regulations of biotechnology in industry, agriculture and medicine. Elsevier, Oxford, pp 933–944. ISBN 0080325122

Walzem R, Dillard C, German J (2002) Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. Crit Rev Food Sci Nutr 42:353-375. doi: 10.1080/10408690290825574

Wang X, Lv M, Zhang L, Li K, Gao C, Ma C, Xu P (2013) Efficient bioconversion of 2,3-butanediol into acetoin using *Gluconobacter oxydans* DSM 2003. Biotechnol Biofuels 6:155. doi: 10.1186/1754-6834-6-155

Wang X-X, Hu H-Y, Liu D-H, Song Y-Q (2016) The implementation of high fermentative 2, 3-butanediol production from xylose by simultaneous additions of yeast extract, Na_2EDTA, and acetic acid. New Biotechnol 33:16-22. doi: 10.1016/j.nbt.2015.07.004

Wendisch VF, Brito LF, Lopez MG, Hennig G, Pfeifenschneider J, Sgobba E, Veldmann KH (2016) The flexible feedstock concept in Industrial Biotechnology: metabolic engineering of *Escherichia coli*, *Corynebacterium glutamicum*, *Pseudomonas*, *Bacillus* and yeast strains for access to alternative carbon sources. J Biotechnol 234:139-157. doi: 10.1016/j.jbiotec.2016.07.022

Wisconsin-University (2015) *E. coli* Genome Project. https://www.genome.wisc.edu/resources/protocols/mopsminimal.htm. Accessed 19 December 2017

Wlaschin KF, Hu W-S (2006) Fedbatch culture and dynamic nutrient feeding. Adv Biochem Eng/Biotechnol 101:43-74. doi:10.1007/10_015

Wong C-L, Huang C-C, Lu W-B, Chen W-M, Chang J-S (2012) Producing 2, 3-butanediol from agricultural waste using an indigenous *Klebsiella* sp. Zmd30 strain. Biochem Eng J 69:32-40. doi: 10.1016/j.bej.2012.08.006

Wong C-L, Yen H-W, Lin C-L, Chang J-S (2014) Effects of pH and fermentation strategies on 2, 3-butanediol production with an isolated *Klebsiella* sp. Zmd30 strain. Bioresour Technol 152:169-176. doi: 10.1016/j.biortecch.2013.10.101

Wu K-J, Saratale GD, Lo Y-C, Chen W-M, Tseng Z-J, Chang M-C, Tsai B-Ch, Su A, Chang J-S (2008) Simultaneous production of 2, 3-butanediol, ethanol and hydrogen with a *Klebsiella* sp. strain isolated from sewage sludge. Bioresour Technol 99:7966-7970. doi: 10.1016/j.biortech.2008.03.062

Wulff E, Mguni C, Mansfeld-Giese K, Fels J, Lübeck M, Hockenhull J (2002) Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. *campestris*. Plant Pathol 51:574-584. doi: 10.1046/j.1365-3059.2002.00753.x

Xiao Z, Lu JR (2014) Strategies for enhancing fermentative production of acetoin: a review. Biotechnol Adv 32:492-503

Xiao Z, Wang X, Huang Y, Huo F, Zhu X, Xi L, Lu JR (2012) Thermophilic fermentation of acetoin and 2, 3-butanediol by a novel *Geobacillus* strain. Biotechnol Biofuels 5:88. doi: 10.1186/1754-6834-5-88

Xin F, Basu A, Weng MC, Yang K-L, He J (2016) Production of 2,3-butanediol from sucrose by a *Klebsiella* species. BioEnergy Res 9:15-22. doi:10.1007/s12155-015-9653-7

Xiu Z-L, Zeng A-P (2008) Present state and perspective of downstream processing of biologically produced 1, 3-propanediol and 2, 3-butanediol. Appl Microbiol Biotechnol 78:917-926. doi: 10.1007/s00253-008-1387-4

Xu H, Jia S, Liu J (2013) Development of a mutant strain of *Bacillus subtilis* showing enhanced production of acetoin. Afr J Biotechnol 10:779-799

Xu Q, Xie L, Li Y, Lin H, Sun S, Guan X, Hu K, Shen Y, Zhang L (2015) Metabolic engineering of *Escherichia coli* for efficient production of (3R)-acetoin. J Chem Technol Biotechnol 90:93-100. doi:10.1002/jctb.4293

Xu Y, Chu H, Gao C, Tao F, Zhou Z, Li K, Li L, Ma C, Xu P (2014) Systematic metabolic engineering of *Escherichia coli* for high-yield production of fuel bio-chemical 2, 3-butanediol. Metab Eng 23:22-33. doi: 10.1016/j.ymben.2014.02.004

Yan Y, Lee C-C, Liao JC (2009) Enantioselective synthesis of pure (R,R)-2,3-butanediol in *Escherichia coli* with stereospecific secondary alcohol dehydrogenases Organic Biomol Chem 7:3914-3917 doi:10.1039/B913501D

Yang T, Rao Z, Zhang X, Lin Q, Xia H, Xu Z, Yang S (2011) Production of 2, 3-butanediol from glucose by GRAS microorganism *Bacillus amyloliquefaciens*. J Basic Microbiol 51:650-658. doi: 10.1002/jobm.201100033

Yu H, Perni S, Shi JJ, Wang DZ, Kong MG, Shama G (2006) Effects of cell surface loading and phase of growth in cold atmospheric gas plasma inactivation of *Escherichia coli* K12. J Appl Microbiol 101:1323-1330 doi:10.1111/j.1365-2672.2006.03033.x

Yu M, Huang M, Song Q, Shao J, Ying X (2015) Characterization of a (2R,3R)-2,3-butanediol dehydrogenase from *Rhodococcus erythropolis* WZ010. Molecules 20:7156. doi:10.3390/molecules20047156

Zeng A-P, Biebl H (2002) Bulk chemicals from biotechnology: the case of 1, 3-propanediol production and the new trends. Adv Biochem Eng Biotechnol 74:239-259. doi: 10.1007/3-540-45736-4_11

Zwietering MH, Jongenburger I, Rombouts FM, van 't Riet K (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol 56:1875-1881

Annex 1. Fermentation of glucose and galactose by a genetically modified strain of *Escherichia coli* into acetoin and 2,3-butanediol

Avant-propos:

L'article "Fermentation of glucose and galactose by a genetically modified strain of *Escherichia coli* into acetoin and 2,3-butanediol" sera soumis en tant que compte-rendu de conférence au cours de la prochaine année

TITRE: Fermentation du glucose et du galactose par une souche génétiquement modifiée d'*Escherichia coli* afin de produire de l'acétoïne et de 2,3-butanediol

Title: Fermentation of glucose and galactose by a genetically modified strain of *Escherichia coli* into acetoin and 2,3-butanediol

Contribution to the document: This paper shows the potential of a genetically modified strain of *Escherichia coli* (*E. coli* JFR1) to transform glucose and galactose into ABD (acetoin (A) and 2,3-butanediol (2,3-BD)). In this study, the differences between glucose and galactose as a carbon source for the ABD production are shown, presenting the ability of *E. coli* to consume the monosaccharides of lactose and the potential of the mutant strain to valorize the disaccharide.

Fermentation of glucose and galactose by a genetically modified strain of *Escherichia coli* into acetoin and 2,3-butanediol

A.1 Résumé

L'acétoïne (A) et le 2,3-butanediol (2,3-BD) sont deux produits chimiques importants car ils permettent entre autres de générer des produits chimiques comme le 1,3-propanediol. Leur production peut être réalisée par voie biologique en présence de microorganismes, producteurs naturels d'A et de 2,3-BD. Néanmoins, le principal problème de ces bactéries telles que *Klebsiella pneumoniae* et *K. oxytoca* pour la production d'ABD (A + 2,3-BD) est leur niveau de biosécurité car elles sont pathogènes. Par contre, des bactéries non pathogènes peuvent être génétiquement modifiées pour produire de l'ABD. Dans cette étude, une souche génétiquement modifiée d'*Escherichia coli* K12 MG1655 a été utilisée afin de produire de l'ABD. Deux monosaccharides (glucose et galactose) sous trois concentrations (12.5, 25 et 50 g/L) ont été fermentés pendant 120 h à 37ºC, 1 atm, pH initial de 7.4, 100 rpm et 10% (v/v) d'inoculum dans un milieu de culture synthétique (M9). Toutes les expériences réalisées ont montré que la fermentation du galactose était moins efficace que celle du glucose (les rendements en ABD ont été de 28, 37 et 43% (concentration de galactose de 12.5, 25 et 50 g/L) inférieurs à ceux obtenus lors de la fermentation du glucose. Le rendement le plus élevé en ABD a été 0.25 g/g de glucose, lequel a été obtenu à 72 h pour une concentration de glucose de 25 g/L.

Mots-clefs: Monosaccharides, Escherichia coli, milieu de culture M9, biovalorisation.

A.2 Abstract

Acetoin (A) and 2,3-butanediol (BD) are two important chemical since they can be transformed into other chemical products like 1,3-propanediol. Their production can be performed by biological way in the presence of microorganisms, natural producers of A and 2,3-BD. However, the main issue of these bacteria to produce ABD (A + 2,3-BD), such as *Klebsiella pneumoniae* or *K. oxytoca*, is their biosafety level since they are pathogen. In this way, non-pathogenic bacteria can be genetically modified to produce ABD. In the present study, a genetically modified strain of *Escherichia coli* K12 MG1655 (non-pathogenic strain) was used in order to produce ABD. Two monosaccharides (glucose and galactose) at three concentrations (12.5, 25 and 50 g/L) were fermented for 120 h at 37ºC, 1 atm, initial pH 7.4, 100 rpm and 10% (v/v) of inoculum in a synthetic culture medium (M9). All experiments showed that the fermentation of galactose was less efficient than glucose (the ABD yields were 28%, 37% and 43% at 12.5, 25 and 50 g/L of galactose compared to those obtained fermenting glucose). The highest ABD yield was 0.25 (g ABD/g glucose), obtained at 96 h in the presence of 25 g/L of glucose at 72 h.

Keywords: Monosaccharide, glucose, galactose, M9 culture medium, urea, biovalorisation

A.3 Introduction

Nowadays there is a consciousness-raising about global warming and sustainability and, thus, the search of new green technologies taking a special interest to obtain chemical products coming from natural sources instead of petroleum (Cho et al. 2015). Among these green techniques, fermentation allows to use natural sources like mono-, oligo- and poly-saccharides which are wastes from the agro-food industry (Mazumdar et al. 2013). These saccharides can be transformed into fermentative products like acetoin (A) and 2,3-butanediol (BD) by bacteria like *Enterobacter cloacae*, *Klebsiella oxytoca* or *Serratia marcescens* (Fernández-Gutierrez et al. 2017). However, other bacteria like *Escherichia coli* K12 MG1655 can host the metabolic pathway of BD from natural producers (Mazumdar et al. 2013).

Escherichia coli K12 MG1655 presents several advantages compared to the previous mentioned strains: i) has a biosafety level 1 (non-pathogenic), ii) can consume a wide range of saccharides like glucose and iii) is easily modified to host metabolic pathways from other microorganisms (iGEM 2016; Tong et al. 2016). Among the different substrates which *E. coli* is able to break down are glucose and galactose (monosaccharides). In order to produce A and BD (ABD), *E. coli* has to transform both monosaccharides into pyruvic acid (PA), which occurs by different pathways: glycolysis for glucose and Leloir pathway + glycolysis for galactose (Lazar et al. 2015, Xu et al. 2014). The enzymatic transformation from glucose and galactose into PA is shown in Figure 1. Afterwards, the PA is transformed into α-acetolactate by α-acetolactate synthase (ALS). Then, α-acetolactate is transformed into A by means of α-acetolactate decarboxylase (ALDC) enzyme. Finally, A is converts into BD by 2,3-butanediol dehydrogenase (BDH) enzyme (Xiao and Lu 2014). Figure 2 shows the metabolic pathway to obtain A and 2,3-BD from glucose and galactose.

In the present study, a genetically modified strain of *E. coli* K12 MG1655 (*E. coli* JFR1) was used to host the metabolic pathway of BD from *E. cloacae* in order to test the ability of *E. coli* JFR1 to produce ABD from glucose and galactose. Three monosaccharide concentrations (12.5, 25 and 50 g/L) were fermented at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm and the ABD yields were compared. The culture medium, M9, was used since in preliminary studies and was selected as the best culture medium to obtain ABD from glucose (Fernández-Gutierrez et al. 2018).

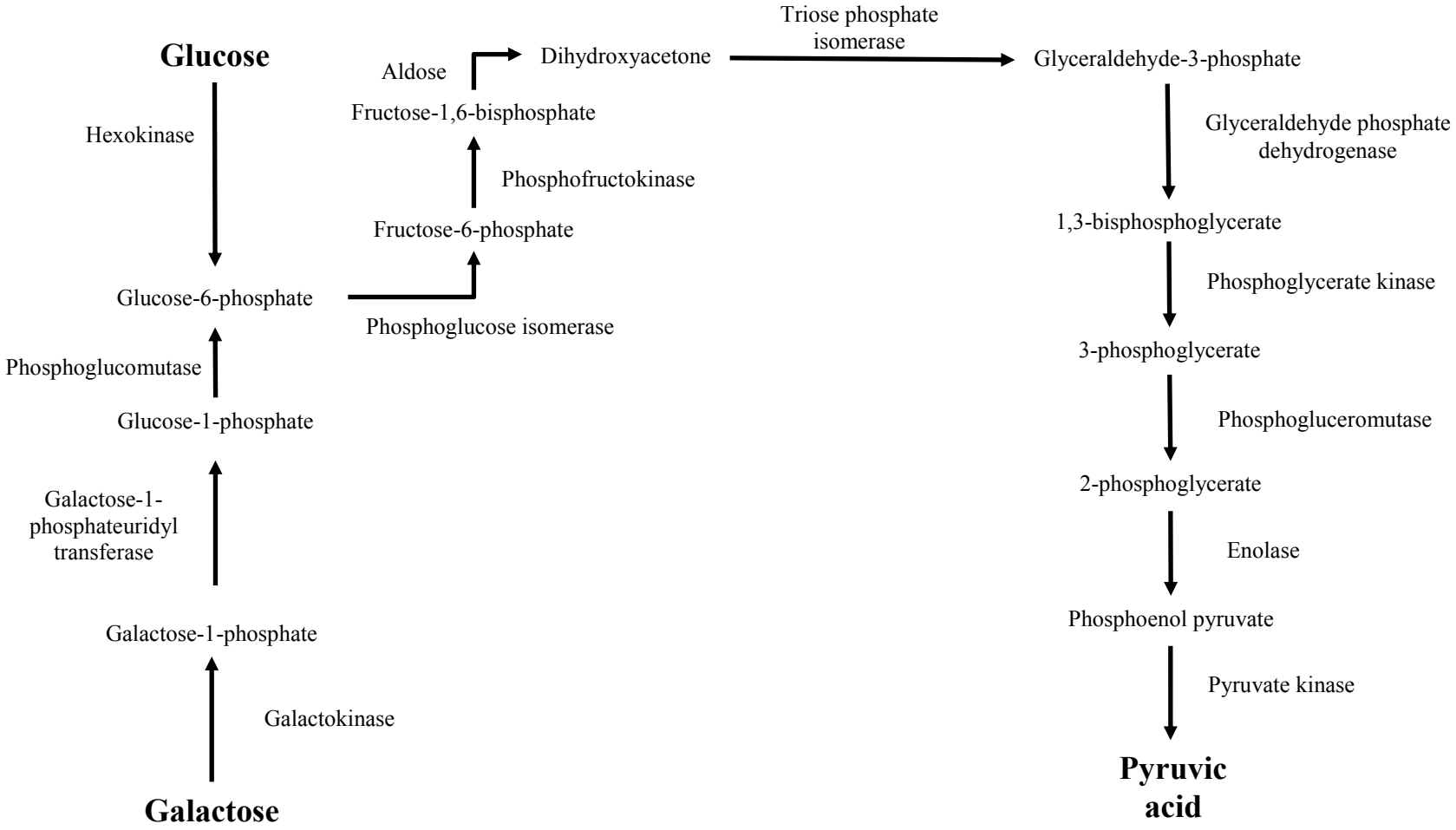


Figure A.1: Scheme of enzymatic reactions involved in the transformation of glucose and galactose into pyruvic acid.

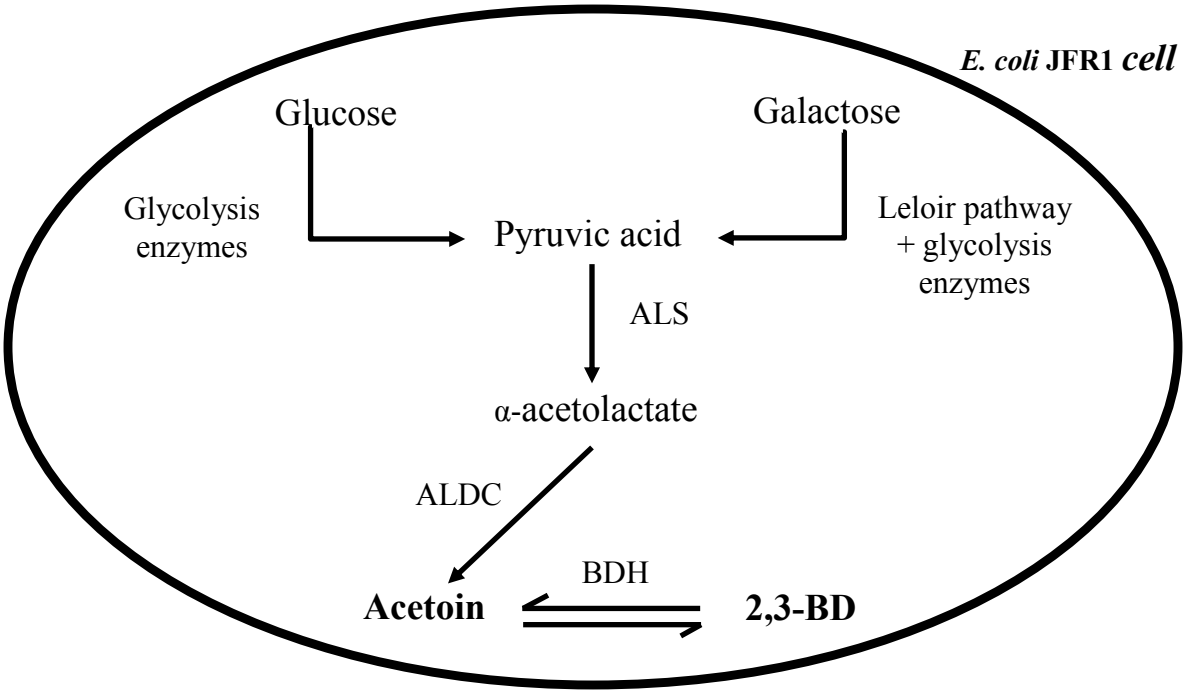


Figure A.2: Metabolic pathway to produce 2,3-BD from glucose and galactose fermentation in the presence of a genetically modified strain of *Escherichia coli*. Relevant reactions to transform pyruvic acid into 2,3-BD are represented by the names of the corresponding enzymes from *Enterobacter cloacae*: α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC) and 2,3-butanediol dehydrogenase (BDH). Sources: Lazar et al. (2015), Mazumdar et al. (2013), Xiao and Lu (2014), Xu et al. (2014), Xu et al. (2015).

A.4 Materials and methods

A.4.1 Microorganism

The genetically modified strain of *E. coli* MG1655 hosts the metabolic pathway of 2,3-butanediol to produce ABD from *Enterobacter cloacae*. The biosynthetic pathway of fermentative D-lactate dehydrogenase (*ldhA*) was blocked to avoid the formation of lactic acid. The modified strain was *E. coli* MG1655/D*ldhA* + *bud*ABC, named as *E. coli* JFR1. The conservation of *E. coli* JFR1 was performed at -81ºC in a blend (50:50, v/v) of glycerol and lysogeny broth (LB) culture medium.

A.4.2 Culture media and conditions

Escherichia coli JFR1 was grown in LB culture medium since it is suitable for recombinant *E. coli* strains (Maniatis et al. 2001). The composition of LB culture medium was as follows: 10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of sodium chloride (NaCl) and

distilled water (Berney et al. 2006). The pH of LB for growing *E. coli* JFR1 was adjusted at 6.5. The fermentation of glucose and galactose by *E. coli* JFR1 was tested using medium 9 (M9), which was made as follows: 12.8 g/L of sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), 3 g/L of potassium dihydrogen phosphate (KH_2PO_4), 1 g/L of ammonium chloride (NH_4Cl), 0.5 g/L NaCl, 15 g/L urea ($(NH_2)_2CO$), 0.49 g/L of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and 0.01 g/L of calcium chloride ($CaCl_2$) and distilled water (Fernández-Gutierrez et al. 2018).

The seed culture medium was prepared as follows: a sample of *E. coli* JFR1 from the conservation blend was taken with a tip and mixed with 10 mL of fresh LB medium in a test tube (15 mL). The test tube was incubated overnight at 37ºC (pre-culture medium). Then, 2 mL of the pre-culture medium were transferred into a 0.5 L Erlenmeyer flask containing 0.2 L of fresh LB medium and incubated in a rotary shaker incubator (Fermentation Design inc, Allentown, PA) at 37ºC, 1 atm, initial pH 6.5 and 100 rpm to reach a bacterial population of $6.5x10^8$ colony-forming unit (CFU)/mL in 9 h. The seed culture medium was used to inoculate M9.

All fermentations were performed in triplicate at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm in 0.5 L flask (0.2 L of working volume).

A.4.3 Analytical methods and reagents

The analysis of glucose, galactose, BD and A was determined by high performance liquid chromatography (HPLC) as shown in previous studies (Fernández-Gutierrez et al. 2018).

A.4.4 Statistical analysis

The ABD formation may be influenced by the type and concentration of the substrate. In this way, the effect of glucose and galactose concentration on ABD yield was statistically determined by means of an analysis of variance (ANOVA) at $p < 0.05$. In addition, Dixon's Q test was carried out to estimate and rule out the outlier values of ABD yield at a confidence level of 95% (Rorabacher 1991).

A.5 Results and discussion

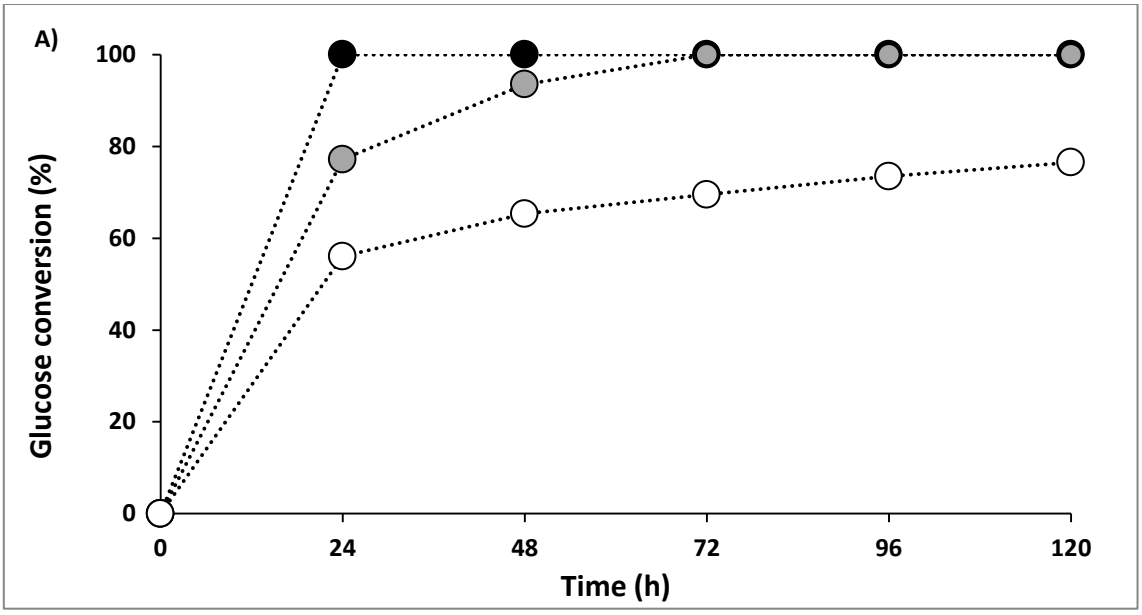
The fermentation of glucose and galactose was performed in M9 culture medium in order to produce ABD by *E. coli* JFR1. The effect of 3 concentrations (12.5, 25 and 50 g/L) of glucose and galactose were tested.

Figure A.3 (A and B) shows the glucose and galactose conversion for 3 initial concentrations (12.5, 25 and 50 g/L) of glucose and galactose ($[Glu]_0$ and $[Gal]_0$, respectively) as a function of time. At 24 h, for a $[Glu]_0$ of 12.5 g/L, the glucose conversion was 100%, whereas for a $[Glu]_0$ of 25 and 50 g/L conversions were 77% and 56%, respectively. Glucose conversion was 100% for a $[Glu]_0$ of 25 g/L at 72 h, while, the

conversion was 70% fermenting 50 g/L. The maximum conversion for a $[Glu]_0$ of 50 g/L was 77%, obtained at 120 h (Figure A.3A).

Similarly, the galactose conversion was 100% for a $[Gal]_0$ of 12.5 g/L at 24 h; whereas it was 75 and 55% in the presence of 25 and 50 g/L of galactose, respectively. At 72 h, the galactose conversion was 100% using a $[Gal]_0$ of 25 g/L and it was 72% for a $[Gal]_0$ of 50 g/L. The maximum galactose conversion for a $[Gal]_0$ of 50 g/L was 76%, which was reached at 120 h (Figure A.3B).

As observed in Figure A.3A and B, the trend of glucose and galactose conversions was similar for both monosaccharides whatever the concentration used. On the other hand, the use of a high concentration (50 g/L) led to a low conversion (around 75%) even after 120 h of fermentation. Bacteria present a threshold concentration to which the bacterial population might be affected if it is exceeded, causing a slower bacterial growth as suggested by Chan et al. (2016). In the case of *E. coli* JFR1, this threshold concentration seems to be between 25 and 50 g/L since the 100% conversion was reached using 25 g/L for both monosaccharides at 72 h; however, it was not achieved for 50 g/L at 120 h.



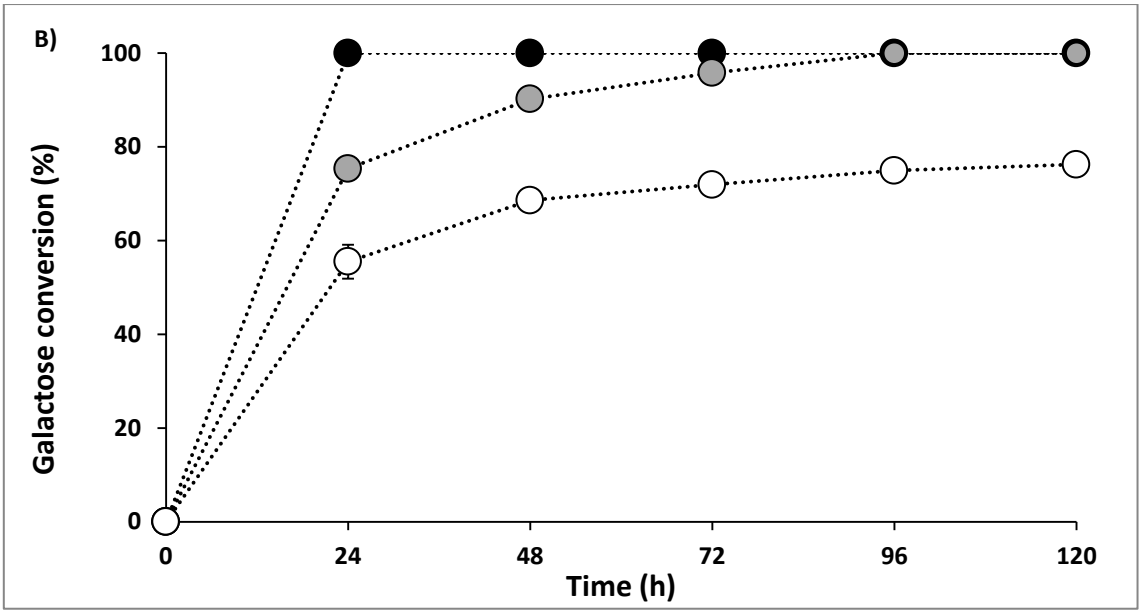


Figure A.3: Glucose (Figure A.3A) and galactose (Figure A.3B) conversion as a function of time. Results are means ± SD of 3 replications performed in flasks at 37ºC, 1 atm, initial pH 7.4, 100 rpm and 10% (v/v) of inoculum in the presence of *E. coli* JFR1 in M9 culture medium (200 mL of working volume) with 12.5 (●), 25 (●) and 50 (○) g/L of monosaccharide.

Figure A.4 (A and B) shows the ABD yield for 3 initial concentrations (12.5, 25 and 50 g/L) of glucose and galactose ($[Glu]_0$ and $[Gal]_0$, respectively) as a function of time. For the $[Glu]_0$ of 12.5 g/L, the maximum ABD yield (0.18 g/g glucose) was obtained at 24 h and remained nearly constant up to 120 h. For a $[Glu]_0$ of 25 g/L, the ABD yield increased reaching a plateau at 0.25 g/g glucose at 72 h; whereas with the $[Glu]_0$ of 50 g/L, the ABD yield increased up to 0.15 g/g glucose at 120 h ($p < 0.05$) as shown in Figure A.4A.

In the case of galactose, for a $[Gal]_0$ of 12.5 g/L, the ABD yield reached a plateau at 0.13 g/g galactose at 24 h (Figure A.4B). Using a $[Gal]_0$ of 25 g/L, the ABD yield increased up to 0.15 g/g galactose ($p < 0.05$) at 72 h and remained constant until the end of the experiment (120 h); whereas in the presence of $[Gal]_0$ of 50 g/L, the ABD yield increased to 0.08 g/g galactose at 72 h and remained nearly constant up to 120 h ($p < 0.05$).

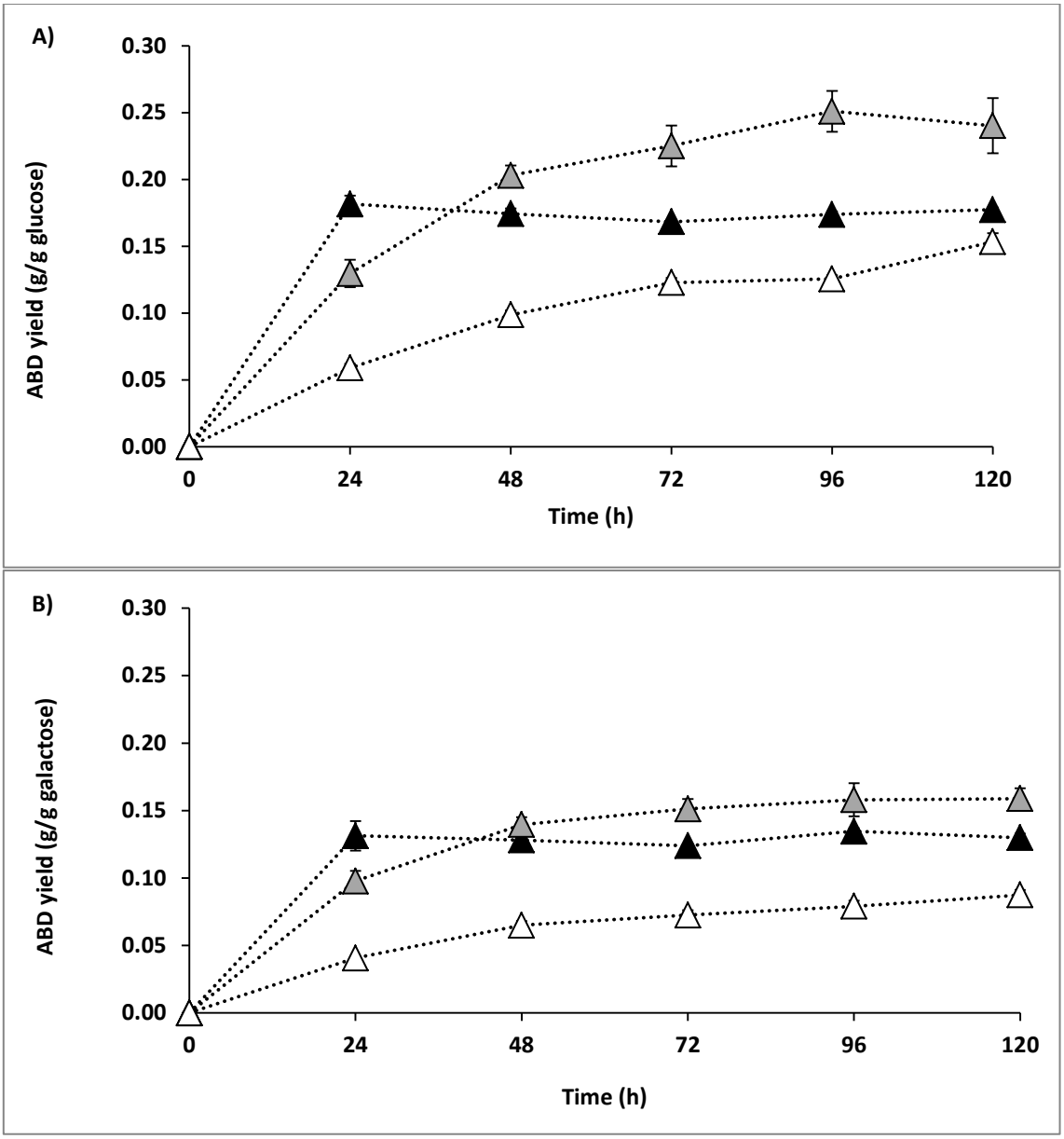


Figure A.4: ABD yield from glucose (Figure A.4A) and galactose (Figure A.4B) as a function of time. Results are means ± SD of 3 replications performed in flasks at 37ºC, 1 atm, initial pH 7.4, 100 rpm and 10% (v/v) of inoculum in the presence of *E. coli* JFR1 in M9 culture medium (200 mL of working volume) at 12.5 (▲), 25 (▲) and 50 (Δ) g/L of monosaccharide.

Comparing the 3 glucose concentrations, the maximum ABD yield was 0.25 g/g glucose at 72 h in the presence of 25 g/L of glucose, which was 28% and 39% higher compared to the maximum obtained using 12.5 and 50 g/L of glucose, respectively. In the case of galactose, the highest ABD yield (0.15 g/g galactose) was reached in the presence of a $[Gal]_0$ of 25 g/L at 72 h. However, this ABD yield (0.15 g/g galactose) was only 13% higher compared to the one obtained with 12.5 g/L of galactose (0.13 g/g galactose), obtained at 24 h, a

fermentation time 3 fold shorter. Therefore, the galactose concentration that gave an ABD yield of 0.13 g/g galactose at a short fermentation time (24 h) was 12.5 g/L.

The difference between both monosaccharides in terms of ABD yield is that the use of glucose was more efficient than galactose since the ABD yields were 28%, 40% and 47% higher using glucose at 12.5, 25 and 50 g/L compared to galactose. As mentioned previously, the use of a high monosaccharide concentration (50 g/L) might lead to the inhibition of the bacterial growth, which affects the formation of ABD as suggested by Chan et al. (2016), Krämer (2010) and Priya et al. (2016). This phenomenon is clearly observed in the present study at 50 g/L of monosaccharide in the presence of *E. coli* JFR1 since the ABD yield at a $[Glu]_0$ of 25 g/L was 40% higher than the one obtained at a $[Glu]_0$ of 50 g/L; the ABD yield was 50% higher at a $[Gal]_0$ of 25 g/L than at 50 g/L of galactose.

As observed in Figure A.4 (A and B), the ABD yield reached a maximum and was nearly constant whatever the monosaccharide and concentration used. As shown in Figure 2, the diol (BD) is an end product which is in equilibrium with A, its precursor. Acetoin is transformed into BD by the BDH enzyme. Mazumdar et al. (2013) suggested that BD can be used by *E. coli* and, thus, can be transformed into A. This might explain why using *E. coli* JFR1, the ABD yield was nearly constant in the present study.

To the best of our knowledge, *E. coli* has not been used to ferment glucose and galactose in order to obtain ABD. However, other studies reported a comparison of glucose and galactose in the presence of bacteria like *Enterobacter aerogenes*, *Geobacillus*, *Paenibacillus polymyxa* and *Saccharomyces cerevisiae* (Choi et al. 2016, Jiang et al. 2015, Jung et al. 2012, Xiao et al. 2012). For example, Xiao et al. (2012) reported an ABD yield of 0.34 and 0.21 g/g monosaccharide (ABD yield calculated from the data provided by the authors) fermenting 20 g/L of glucose and galactose respectively at 55ºC and 170 rpm (pH and time non-defined) in the presence of *Geobacillus* sp. XT15 (5% (v/v) of inoculum). Although the yields were superior to those obtained in the present study, the difference of ABD yield between both monosaccharides was 1.6 fold higher using glucose than galactose; a similar difference in terms of ABD yield in the presence of *E. coli* JFR1 using 25 g/L of monosaccharide at 96 h was observed. Therefore, the use of glucose seems to be more suitable than galactose in order to produce ABD in the presence of *E. coli* JFR1.

A.6 Conclusion

The present study was based on the fermentation of 2 monosaccharides in order to produce acetoin and 2,3-butanediol (ABD) in the presence of a genetically modified strain of *Escherichia coli*: *E. coli* JFR1. Different concentrations (12.5, 25 and 50 g/L) of glucose and galactose in M9 culture medium at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm in 0.5 L flask (0.2 L of working volume) were fermented.

The use of whatever glucose concentration provided better results in terms of ABD yield than the fermentation of galactose. The ABD yields were between 28% and 47% higher in the presence of glucose for the range of monosaccharide concentrations tested. The highest ABD yield was 0.25 g/g glucose at 72 h and 25 g/L of glucose; whereas the best ABD yield was 0.13 g/g galactose at 24 h and 12.5 g/L of galactose.

This study demonstrated that galactose can be used by *E. coli* JFR1 in order to produce ABD. Hence, an in depth study using this monosaccharide should be considered in the presence of *E. coli* JFR1 with the aim of improving the ABD yield.

A.7 Acknowledgement

Michèle Heitz is grateful to Fond Québécois de la Recherche sur la Nature et les Technologies (FRQNT) in partnership with MAPAQ-NOVALAIT (Innovation en Production et en Transformation Laitières) for the grant attributed to the research program "Développement d'une biotechnologie verte innovatrice pour valoriser des résidus fromagers". The authors are grateful to the laboratory technicians (Department of chemical and biotechnological engineering of Université de Sherbrooke) Isabelle Arsenault and Valérie Larouche for their support and help. The authors would like to particularly thank Pr. Sébastien Rodrigue and the master student Jean-François Rousseau (Department of biology of Université de Sherbrooke) for providing the genetically modified strain *E. coli* JFR1. Michèle Heitz would also like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for a financially contribution at the end of the study.